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**INVESTIGATING TUMOR SUPPRESSORS IN THE DNA DAMAGE
RESPONSE: CARETAKERS OF THE GENOME AND BIOMARKERS TO
PREDICT THERAPEUTIC RESPONSE**

A Dissertation Presented

By

SHAWNA SUSAN GUILLEMETTE

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

April 11th, 2014

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DEDICATION

I want to dedicate this dissertation to my family and friends that were lost along the way. To my paternal Grandfather who recently passed to Parkinson's disease and to my maternal Grandfather who was taken by cancer at age 48 and unable to meet any of his grandchildren. To my cousin Christopher Boucher and my childhood friends Matthew Dickson and Patrick McGovern who have been with me in spirit throughout this long journey.

ACKNOWLEDGEMENTS

I would like to first thank my mentor Sharon Cantor for her continuous encouragement and support throughout this entire journey. There were times when I was frustrated trouble shooting an experiment and nearly wanted to give up and pursue a different direction. In those times Sharon encouraged me the most, even when I did not have confidence in myself. My favorite quote from Sharon is *“there is nothing wrong with your hands, go back to the bench and start again!”* Sure enough, these encouraging words always eventually led to success with working controls and meaningful data that I appreciated more than any other experiment. Sharon also taught me that being successful in science is not just about intelligence or perseverance, but also requires confidence. Because of this I am a better scientist and better at communicating my own research findings with the scientific community.

This work was launched with the help of several lab members. I want to thank Jenny Xie, Min Peng, and Rachel Litman for all of their help and ongoing support. Their previous studies paved the way for my investigation of FANCD1 in the response to UV irradiation. There were also several valuable reagents made by these members and I was grateful to use them in my own research.

I want to thank Orlando Schärer for his insightful discussions on the DNA damage response to UV irradiation and expertise in the field of Nucleotide Excision Repair. His lab generously provided the Xeroderma Pigmentosum patient derived cells lines used to examine FANCD1 recruitment in chapter II.

I want to acknowledge and thank Michael Green and his former graduate student Ryan Serra. Michael generously provided the library used for my shRNA screen described in chapter III. It was the first time that our lab and myself ever performed an shRNA screen and without his help and generosity the project would never have taken off. I am also especially grateful for Ryan who has been my role model and taught me to value the importance of optimization prior to launching a screen. Aside from the screen, nearly every technique I use today from viral particle production to bacterial colony PCR to chromatin preparation has improved because of Ryan's constructive criticism and guidance. I am also thankful for his ongoing friendship and enlightening scientific discussions.

I also want to thank my family who have provided constant support throughout my research endeavors. I especially want to thank my grandmothers, my parents, and my brother Derek. I want to thank my close friends the Shannis family, the Pieroni family, and the Muse family for all of their encouragement over the years. I am so thankful and fortunate to have a loving and supportive community close to me in New Hampshire where I could visit and recharge my soul for the long road ahead. Although my parents are not scientists, their enthusiasm for nature and the outdoors undeniably propelled my interest in science from an early age. For that, I am forever thankful.

ABSTRACT

Our genome is constantly challenged by sources that cause DNA damage. To repair DNA damage and maintain genomic stability eukaryotes have evolved a complex network of pathways termed the DNA damage response (DDR). The DDR consists of signal transduction pathways that sense DNA damage and mediate tightly coordinated reactions to halt the cell cycle and repair DNA with a collection of different enzymes. In this manner, the DDR protects the genome by preventing the accumulation of mutations and DNA aberrations that promote cellular transformation and cancer development. Loss of function mutations in DDR genes and genomic instability occur frequently in many tumor types and underlie numerous cancer-prone hereditary syndromes such as Fanconi Anemia (FA).

My thesis research applies candidate-based and unbiased experimental approaches to investigate the role of several tumor suppressor genes (TSGs) in the DDR. My dissertation will first describe a novel function for the breast and ovarian cancer tumor suppressor and FA-associated gene FANCD1 in the DDR to ultraviolet (UV) irradiation. In response to UV irradiation FANCD1 supports checkpoint induction, the arrest of DNA synthesis, and suppresses UV induced point mutations. Suggesting that FANCD1 could suppress UV induced cancers, in sequenced melanomas from multiple databases I found somatic mutations in FANCD1 previously associated with breast/ovarian cancer and FA syndrome.

The second part of my dissertation will describe an RNA interference

screen to identify genes modulating cellular sensitivity to the chemotherapeutic drug cisplatin. The hereditary breast/ovarian cancer tumor suppressor BRCA2 is essential for DNA repair, thus BRCA2 mutant ovarian cancer cells are initially sensitive to cisplatin chemotherapy that induces DNA damage. However, drug resistance develops and remains a major problem in the clinic. My screen identified the chromatin remodeling factor CHD4 as a potent modulator of cisplatin sensitivity and predictor of response to chemotherapy in BRCA2 mutant cancers. Taken together, my investigations highlight the important contribution of the DDR and the role they play in tumorigenesis and predicting therapeutic response.

TABLE OF CONTENTS

Title Page.....	I
Signature Page.....	II
Dedication.....	III
Acknowledgements.....	IV
Abstract.....	VI
Table of Contents.....	VIII
List of Tables.....	IX
List of Figures.....	X
List of Publications.....	IX
Chapter I: Introduction.....	1
Chapter II: FANCDJ function in the DNA Damage Response to UV Irradiation....	34
Preface.....	34
Abstract.....	36
Introduction.....	37
Results.....	40
Figures	50
Conclusions	83
Methods.....	89
Chapter III: Cisplatin resistance screen in BRCA2 mutant Cells	97
Preface.....	97
Abstract.....	98
Introduction.....	99
Results.....	102
Figures.....	111
Conclusions.....	144
Methods.....	152
Chapter IV: Final Summary and Discussion.....	157
Bibliography.....	167

LIST OF TABLES

1-1 List of Human genetic diseases associated with defects in DNA damage response	30
2-1 List of UV-induced mutations at <i>HPRT</i> locus	76
2-2 List of Mutations in <i>FANCI</i> and MMR loci from melanoma genomes	80
3-1 List of <i>BRCA2</i> ovarian cancer mutations used for Kaplan-Meier analysis	139
3-2 List of candidates identified in shRNA screen in PEO1 cells	142

LIST OF FIGURES

1-1 Schematic of the DNA damage response in the context of chromatin	24
2-1 FANCD1 recruitment to sites of local UV-induced damage (LUDs)	50
2-2 FANCD1 recruitment to LUDs is dependent on XPA	51
2-3 FANCD1 recruitment to LUDs is dependent on XPD catalytic activity	52
2-4 FANCD1 recruitment to LUDs is dependent on XPG catalytic activity	53
2-5 FANCD1 focal accumulation in response to global UV-irradiation is dependent on XPD catalytic activity	54
2-6 NER factors are recruited to sites of LUDs independently of FANCD1	55
2-7 NER dependent FANCD1 recruitment to LUDs occurs predominantly in S phase	56
2-8 FANCD1 recruitment to LUDs is dependent on MLH1 interaction	57
2-9 FANCD1 recruitment to LUDs is dependent on mismatch repair factors MLH1 and MSH2	58
2-10 FANCD1 recruitment to LUDs is dependent on the mismatch repair factor MSH6	59
2-11 MMR and NER factors are independently recruited to sites of LUDs	60
2-12 Residual FANCD1 accumulation at LUDs in <i>XP-F</i> cells during S phase is eliminated by depletion of MSH2	61
2-13 MSH2 depletion does not perturb FANCD1 chromatin localization	62
2-14 FANCD1 co-localizes with UV induced LUDs predominantly in S phase	63
2-15 FANCD1 contributes to the arrest of DNA synthesis in response to UV irradiation	64
2-16 FANCD1 contributes to phosphorylation of RPA	65
2-17 FANCD1 contributes to phosphorylation of RPA during S phase	66
2-18 FANCD1 contributes to the UV induced checkpoint	67
2-19 FANCD1 contributes to the UV induced checkpoint and elimination of UV induced lesions	68

2-20 FANCI and MMR function in a common pathway for RPA phosphorylation and 6-4 Photoproduct elimination	69
2-21 FANCI and MSH2 are required for RPA phosphorylation in S phase	70
2-22 FANCI and MSH2 are not required for NER dependent gap filling	71
2-23 FANCI and MMR factors are not required for NER dependent gap filling	72
2-24 FANCI precipitates with UV modified PCNA and CPD from chromatin extracts	73
2-25 FANCI deficient cells are sensitive to the DNA interstrand crosslinking agent cisplatin	74
2-26 FANCI suppresses UV-induced mutations	75
2-27 FANCI suppresses UV-induced mutations	77
2-28 FANCI functions in response to UV irradiation	78
2-29 Mutations in <i>FANCI</i> occur in melanoma genomes	79
2-30 Ectopic expression of catalytic inactive FANCI ^{K52R} disrupts clearance of UV induced lesions in U2OS cells	81
2-31 FANCI expression promotes UV induced GFP-pol η foci formation in U2OS cells	82
3-1 PEO1 cells express nuclear truncated BRCA2 and are sensitive to DNA damaging agents compared to BRCA2 revertant C4-2 cells	111
3-2 An RNA interference screen to identify mediators of cisplatin resistance in BRCA2 mutant cells	112
3-3 An RNA interference screen identifies CHD4 as a mediator of cisplatin resistance in BRCA2 mutant cells	113
3-4 Depletion of the nucleosome remodeling (NuRD) complex member MBD2 does not mediate cisplatin resistance	114
3-5 An RNA interference screen identifies CHD4 as a mediator of cisplatin resistance in BRCA2 mutant cells	115
3-6 Cell cycle profiles of BRCA2 restored C4-2 cells and BRCA2 mutant PEO1 cells depleted of CHD4 verses control in response to cisplatin	116

3-7 CHD4 depletion in PEO1 cells does not restore RAD51 foci formation	117
3-8 CHD4 depletion in PEO1 cells does not restore full length BRCA2	119
3-9 CHD4 depletion causes cisplatin sensitivity in BRCA2 proficient cells	120
3-10 CHD4 depleted BRCA2 proficient cells are sensitive to DNA damage while BRCA2 mutant PEO1 cells are partially resistant	121
3-11 CHD4 depletion is toxic in BRCA2 proficient cells, yet uniquely tolerated in BRCA2 mutant cells	122
3-12 CHD4 depletion is toxic in BRCA2 proficient cells, yet uniquely tolerated in BRCA2 mutant cells	123
3-13 CHD4 depletion mediates toxicity in BRCA1 mutant cells	124
3-14 CHD4 depletion mediates toxicity in BRCA1 mutant cells	125
3-15 CHD4 depletion does not mediate cisplatin resistance in other DNA repair deficient cells	126
3-16 CHD4 depleted PEO1 cells are sensitive to the HDAC inhibitor Trichostatin A (TSA)	127
3-17 Co-depletion of CHD4 and BRCA2 in BRCA2 proficient C4-2 cells does not mediate cisplatin resistance	128
3-18 Co-depletion of CHD4 and BRCA2 in BRCA2 proficient U2OS cells does not mediate cisplatin resistance	129
3-19 Cisplatin resistance mediated by CHD4 depletion in PEO1 cells requires expression of truncated BRCA2	130
3-20 Cisplatin resistant clones retain truncated BRCA2 and gain resistance by an unknown mechanism	131
3-21 Cisplatin resistant clones do not restore RAD51 foci formation	132
3-22 Cisplatin resistant clones have reduced CHD4 protein expression	133
3-23 Ectopic expression of GFP-CHD4 restores sensitivity to C4-4 cells	134
3-24 Cisplatin resistant clones are uniquely sensitive to Trichostatin A (TSA)	135
3-25 Progression Free Survival (PFS) and Overall Survival (OS) does not correlate with CHD4 mRNA expression in sporadic ovarian cancers	136

3-26 Progression Free Survival (PFS) and Overall Survival (OS) is not improved in BRCA1 mutant ovarian tumors with increased CHD4 mRNA expression	137
3-27 Progression Free Survival (PFS) and Overall Survival (OS) is improved in BRCA2 mutant ovarian tumors with increased CHD4 mRNA expression	138
3-28 Model of cisplatin resistance mechanisms in BRCA2 mutant cells	140
3-29 Partial validation of candidates identified in the shRNA screen	141
3-30 Truncated BRCA2 can interact with RAD51 in a cisplatin inducible manner	143

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FANCD1 localization by mismatch repair is vital to maintain genomic integrity after UV irradiation.

Guillemette S, Branagan A, Peng M, Dhruva A, Schärer OD, Cantor SB.
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An MLH1 mutation links BACH1/FANCD1 to colon cancer, signaling, and insight toward directed therapy.

Xie J, Guillemette S, Peng M, Gilbert C, Buermeyer A, Cantor SB.
Cancer Prev Res (Phila). 2010 Nov;3(11):1409-16. doi: 10.1158/1940-6207

Targeting the FANCD1-BRCA1 interaction promotes a switch from recombination to poleta-dependent bypass.

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CHAPTER I: INTRODUCTION

Genomic Instability Underlies Cancer

The notion that genomic instability is a common underlying defect for nearly all tumor cell types has been well appreciated and investigated for the latter half of the 20th century. However, in 1902, the documentation of chromosome aberrations by German biologist Theodor Boveri nearly went unpublished as its own manuscript and was initially described in a footnote. Boveri doubly fertilized sea urchin eggs to induce multipolar mitoses and generated daughter nuclei that contained missing or additional chromosomes (Boveri 1902). Sadly, his colleagues felt that his findings would not lead to new areas of investigation, and thus, were not worthy of a separate publication. Unshaken, Boveri took it upon himself to continue his investigations and in 1914 published *Concerning the Origin of Malignant Tumours*. This became a visionary paper for its time describing the genetic basis of cancer and included the often quoted statement “*malignant tumours might be the consequence of a certain abnormal chromosome constitution*” (Boveri 1914).

Boveri's theory that cancer could arise from a single cell with scrambled chromosomes was correct and indeed spawned an entire field of investigation that has lasted nearly 50 years and continues to grow. Since Watson and Crick first solved the crystal structure of DNA, the field of genome instability has focused on investigating the factors that maintain the integrity of our genetic

material and how they are linked to cancer (Watson & Crick 1953). The sequencing of the human genome and discovery of RNA interference has now given cancer biologists better tools to interrogate the factors that protect and repair our genome to suppress cancer.

Although there are many hallmark characteristics of cancer cells, an unstable genome is persistently a common and essential defect (Hanahan et al. 2000; Hanahan & Weinberg 2011). The occurrence of additional cancer-like traits such as limitless growth potential, evasion of cell death, and tissue invasion and metastasis all depend on mutational events in the genome to incur such processes. As such, cancer cells often display an increased mutation frequency in their genomes when compared to the genomes of normal healthy cells (Weinberg, 2007 p. 423-424). However, as Boveri noted, cancer cells can also be unstable at the chromosomal level with additional copies of chromosomes, termed aneuploidy, or unstable due to loss of chromosome fragments or entire chromosomes themselves (Weinberg, 2007 pp. 11-13). To make genomic instability even more complicated, DNA double strand breaks (DSBs) can occur on separate chromosomes and incorrectly fuse together to introduce chromosomal translocations. These translocations are ubiquitous in cancer, especially in leukemia, where genes from separate chromosomes are fused together by translocation to create chimeric proteins that offer increased cellular fitness or even resistance to chemotherapy (Weinberg, 2007 pp. 109-115).

Taken together, genome instability contributes to the genetic aberrations required for cancer cell transformation and also serves to drive the disease to more advanced stages.

Cancer is also frequently referred to as a “disease of aging”. This means that the incidence of cancer increases within the older generations of the population. This likely reflects that cancer is a complex disease that requires time for the accumulation of many genetic insults in the right combination for the cell to progress to a diseased state. Exposure to environmental factors such as carcinogens in tobacco smoke or chimney soot can further contribute to the accumulation of mutations and shorten the period of time required for cellular transformation (Weinberg, 2007 pp. 400-402). In addition, the genome is continuously challenged by non-environmental induced errors that arise as byproducts of normal cellular metabolism or DNA replication (Lindahl & Barnes 2000). When time is factored in, it has been estimated that the genome inside a mammalian cell can experience up to 10^5 spontaneous DNA lesions per day (Hoeijmakers 2009).

The DNA Damage Response

Our genome is constantly challenged by sources that cause DNA damage, yet genomic integrity must be maintained in order to dependably transmit our genetic material to our offspring and to protect us from cancer

associated mutations that can compromise our own survival. To cope with DNA damage and maintain genomic stability eukaryotes have evolved a complex interlocking network of pathways termed the DNA damage response (DDR).

The DDR consists of signal transduction pathways that sense DNA damage or replication induced stress and mediate tightly coordinated responses to halt the cell cycle and repair DNA using a collection of different enzymes specific for the type of DNA damage incurred (Harper & Elledge 2007; Jackson & Bartek 2009; Ciccio & Elledge 2010). Proteins involved in DDR have traditionally been categorized as *sensors*, *transducers*, *mediators*, and *effectors* (Niida & Nakanishi 2006; Polo & Jackson 2011). The sensor proteins are responsible for recognizing damaged DNA and often directly recruit the transducer, mediator, or effector proteins to the lesions to propagate the DDR. I will now succinctly describe the best studied *sensor*, *transducer*, *mediator*, and *effector* proteins essential for the DDR.

Sensors. Many sensor proteins have been investigated and characterized based on the types of DNA lesions that they recognize. Proteins of the Mre11-Rad50-Nbs1 (MRN) complex recognize and bind the ends of DNA double strand breaks (DSBs) through the DNA binding domain of MRE11 tethering the broken ends together (Lee & Paull 2005). Upon DNA binding, the architecture of the MRN complex is altered to promote inter-complex association from both DNA

ends and prevent intra-complex interaction (Moreno-Herrero et al. 2005). MRE11 also contains DNA endonuclease and exonuclease activity that can facilitate DNA end processing, termed DNA resection, making the broken DNA a better substrate for other downstream repair factors (Williams et al. 2007).

Adding to the complexity of sensing DNA DSBs, the Ku heterodimer (Ku70 and Ku80) is a toroidal structure that can also rapidly bind to the ends of DNA DSBs (Mahaney et al. 2009). Binding of the Ku heterodimer to DSBs prevents DNA end resection and promotes a more error prone repair pathway termed non-homologous end joining (NHEJ), which will be further described later (Meek et al. 2008; Mahaney et al. 2009).

PARP1 and PARP2 are enzymes that act as molecular sensors for DNA DSBs, DNA single strand breaks (SSBs), abasic sites, and altered DNA bases such as 8-Oxo-Guanine or 3-Methyl-Adenine (Caldecott 2008). Once bound to DNA, PARP1/2 catalyzes the addition of poly (ADP-ribose) sugar chains to histone proteins H1, H2B, and to PARP1 itself (Schreiber et al. 2006). Histone PARylation by PARP1/2 is thought to recruit additional downstream factors that aid in the remodeling of chromatin at the site of damage (Polo et al. 2010; Schreiber et al. 2006; Chou et al. 2010).

Another dangerous threat to the genome that must be sensed are single stranded DNA (ssDNA) lesions that are instigated by replication fork stress and fork stalling. Bulky DNA lesions encountered by the replication machinery can cause the arrest of leading strand synthesis and the formation of ssDNA regions due to the uncoupling of the replicative MCM helicase and DNA polymerase (Byun et al. 2005). These ssDNA regions are immediately coated by the ssDNA binding protein RPA, which serves as a platform to generate a signaling cascade (Byun et al. 2005). The RPA-ssDNA complex stimulates binding and activation of the RAD17-RFC2-5 DNA clamp loader which in turn recruits proteins of the 9-1-1 complex (RAD9, RAD1, and HUS1) that initiate a signal transduction cascade to stabilize and repair the damage and eventually restart the replication fork (Paulovich et al. 1998; Ellison & Stillman 2003; Cimprich & Cortez 2008).

Finally, the DNA mismatch repair (MMR) family of proteins serves to sense and recognize non-Watson-Crick base pairing (DNA mismatches) that result from misincorporated deoxyribonucleic acids (dNTPs). The MMR family also senses erroneous insertion or deletion loops that form from replication errors (Kunkel & Erie 2005). The MMR complex MSH2-MSH6 has been shown to continually scan DNA searching for errors and is unique in that it can discriminate between the parental and daughter strands of newly replicated DNA (Gorman et al. 2007). Once MMR complexes detect damaged DNA a checkpoint response is mounted to halt the cell cycle and repair the error (Hsieh & Yamane 2008).

Transducers. Once the damage is recognized transducer kinases affect the activity of downstream mediator and effector proteins by regulating phosphorylation and dephosphorylation events. Several transducer proteins of the phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family, including Ataxia-Telangiectasia mutated (ATM) kinase, Ataxia-Telangiectasia and Rad-3-related (ATR) kinase, and DNA Protein Kinase (DNA-PK) facilitate signal transduction pathways that trigger a tightly regulated response to stall the cell cycle and repair specific forms of damage (Ciccio & Elledge 2010). Some crossover exists between these pathways, especially the ATM and ATR kinase pathways. When one pathway is partially or totally deficient that other pathway can stand in to trigger the signal transduction cascade (Bartek & Lukas 2003).

The ATM kinase is initially recruited by the MRN sensor complex to DNA DSBs and has hundreds of protein substrates that amplify the DDR (Uziel et al. 2003; Lee & Paull 2005; Lavin 2007). The ATR kinase forms a complex with its binding partner ATRIP and is recruited to RPA-ssDNA nucleofilaments at sites of stalled replication forks or at RPA-ssDNA regions generated during DSB repair (Cortez et al. 2001; Rouse & Jackson 2002; Zou & Elledge 2003; Cimprich & Cortez 2008). Lastly, the kinase DNA-PK is recruited by the Ku heterodimer to DNA DSBs and is more focused on the recruitment and regulation of proteins involved in DNA end joining to initiate an error prone NHEJ (Mahaney et al.

2009). DNA–PK complex members are also recruited to chromosomal ends known as telomeres, where they function in capping chromosome ends to prevent them from being mistaken for double-strand breaks (Burma & Chen 2004).

Effectors. Transducer kinases also phosphorylate effector kinases. The transducer kinase ATM activates the effector kinase CHK2 in response to DNA DSBs. Similarly, the transducer kinase ATR activates the effector kinase CHK1 in response to bulky DNA lesions or replication stress (Bartek & Lukas 2003; Shiloh 2003). These effectors in turn spread the phosphorylation signal throughout the nucleus to amplify the DDR.

The tumor suppressor protein p53 is a major downstream effector of the ATM/CHK2 DNA damage kinase pathway. The p53 protein functions to induce cell cycle arrest, apoptosis, or senescence in response to genomic insults by transcriptionally regulating inhibitors of cyclin dependent kinases such as p21 (Zhou & Elledge 2000; Meek 2004; Harris & Levine 2005). When DNA damage occurs in normal cells, p53 dependent signaling promotes arrest in the G1 cell cycle phase by transcriptionally up-regulating p21 (Vogelstein et al. 2000). This mechanism provides time to allow DNA repair and blocks entry into synthesis phase. Additionally, p53 mediated up-regulation of p21 can block cells post replication in G2 phase in response to ionizing radiation (Bunz 1998).

Alternatively, if the DNA damage is too extensive p53 can up-regulate pro-apoptotic genes such as BAX and PUMA to initiate cell death (Riley et al. 2008). It is therefore not surprising that p53 is commonly mutated in the majority of tumor types leading to loss of checkpoints and the evasion of cell death (Vogelstein et al. 2000).

Mediators. Mediator proteins assist DNA damage signaling by fostering protein-protein interactions between components of the DDR, effector proteins, or with factors that promote the physical repair of the DNA lesion. A common feature of mediator proteins is BRCA1 carboxyl terminal (BRCT) domains, which function as protein-phosphoprotein interaction modules (Manke et al. 2003; Yu et al. 2003). In response to DNA damage induced signaling, the mediator proteins help recruit the many physical repair pathways that evolved to cope with specific types of DNA damage. I will briefly summarize the well characterized DNA repair pathways recruited to DNA damage by mediator proteins and the different forms of damage that they are best suited to repair.

In response to DNA SSBs or small base adducts such as alkylation products or oxidative damage the Base Excision Repair (BER) pathway is recruited. BER is a multistep process that first removes the damaged bases from one strand of the DNA helix by excision and then replaces the excised backbone with newly synthesized DNA bases. The poly (ADP-ribose) polymerase enzymes

PARP1 and PARP2 mentioned earlier in this section are essential sensors and signal transducers to initiate this process (Memisoglu & Samson 2000; David et al. 2007).

In contrast, bulky single strand DNA lesions that distort the DNA helix, such as those created by ultraviolet (UV) irradiation, are processed by Nucleotide Excision Repair. NER is traditionally classified into two sub-pathways, transcription-coupled NER, which is activated when the RNA polymerase encounters a DNA lesion, and global-genome NER, which is activated due to disrupted base pairing and general helix distortion. These NER sub-pathways utilize different methods to detect lesions, however the mechanism of repair is very similar. Like BER, the DNA surrounding the lesion on one strand is excised. The endonucleases XPF and XPG, which are mutated in the skin cancer prone disease Xeroderma Pigmentosa, are essential for this excision process. The excised DNA is then filled in using the normal DNA replication machinery, namely polymerase delta, with the aid of additional DNA polymerases (de Boer & Hoeijmakers 2000; Gillet & Schärer 2006).

At DNA lesions that stall replication forks, replication restart can leave ssDNA regions behind the replication fork. These regions are repaired by either Translesion Synthesis (TLS) or error-free Post Replication Repair (PRR), which involves a template switching mechanism. Both pathways are dependent on the

RAD6/RAD18 ubiquitin ligase complex (Branzei & Foiani 2010; Daigaku et al. 2010; Karras & Jentsch 2010). In error prone TLS, the high fidelity DNA replication polymerase is transiently replaced with one of several low fidelity polymerases that contain large active sites to accommodate the lesion and bypass the DNA damage. In the error free PRR template switching mechanism, the single stranded DNA gap generated during replicative bypass is repaired by strand invasion of the sister chromatid which is used as a template for repair (Budzowska & Kanaar 2009). TLS and PRR are both dependent on RAD6/RAD18 mediated monoubiquitination or polyubiquitination, respectively, on lysine 63 of the replicative clamp PCNA (Ulrich & Walden 2010).

The repair of DNA DSBs is more complicated in that several repair pathways have evolved to cope with this type of damage. The pathway of choice is mainly influenced by the cell cycle phase and the extent of DNA end processing. DNA end joining promoted by the Ku heterodimer or PARP proteins proceeds with minimal DNA end processing, is error prone, and typically occurs in G1 phase of the cell cycle prior to duplication of the genome. In classical NHEJ, Ku immediately localizes to DSBs where it recruits and activates the catalytic subunit of DNA-PK. DNA-PK stabilizes DNA ends and prevents resection allowing recruitment of XRCC4/LIG4 which promotes the re-ligation of broken ends with the help of ARTEMIS and the stimulatory factor XLF (Meek et al. 2008; Mahaney et al. 2009). In alternative NHEJ (also known as

microhomology-mediated end joining), which is a backup pathway to classical NHEJ, the PARP proteins compete with Ku at broken DNA ends to promote limited end resection (~5-25 nucleotides) and repair DNA by a mechanism that is similar to homologous recombination which is described next (Wang et al. 2006).

DNA DSBs recognized by the MRN sensory complex activate the signal transduction kinase ATM to process the DNA surrounding the break and promote error free repair by homologous recombination (HR) (Williams et al. 2007). HR mainly occurs during S and G2 phases of the cell cycle and is a conservative process in that it uses a sister chromatid as a template for repair. The first step in HR is the nucleolytic processing of DNA ends by the MRN complex and other enzymes such as the exonuclease EXO1, to promote 5' to 3' DNA end resection (Bernstein & Rothstein 2009; Rupnik et al. 2010). The remaining 3' ssDNA overhangs are coated by RPA and then replaced by RAD51 in a manner directed by the breast cancer associated protein BRCA2. Recent papers describing the purification and biochemical analysis of BRCA2 have shed light on this critical step in HR (Jensen et al. 2010; Liu et al. 2010; Jensen 2013). The RAD51 nucleoprotein filament then initiates a homology search on the sister chromatid and promotes DNA strand invasion forming a D-loop structure. DNA polymerases then synthesize new DNA using the sister chromatid as a template and ligases, helicases, and resolvase enzymes mediate the cleavage and

resolution of HR intermediates to yield repaired DNA molecules (Mazón et al. 2010).

To repair DNA adducts that covalently crosslink the two strands of DNA together and impede replication machinery cells have evolved the Fanconi anemia (FA) pathway. FA was named after the Swiss pediatrician, Guido Fanconi, that discovered the rare, primarily recessive genetic disorder that renders cells extremely sensitive to DNA crosslinking agents such as Cisplatin or Mitomycin C (Moldovan & D'Andrea 2009). The FA pathway consists of at least 16 gene products and is divided into two complexes. Upstream FA proteins are required for the monoubiquitination events of FANCD2 and FANCI proteins, and downstream FA proteins which are not required for ubiquitination, but function at the chromatin level to properly excise the crosslink and restore the DNA using a combination of template switch and HR dependent DNA repair mechanisms previously described (Moldovan & D'Andrea 2009; Kottmann & Smogorzewska 2013).

Adding another layer of complexity to DNA repair is the orderly progression of pathway choice. While the type of lesion or DNA substrate provides some specificity for pathway choice this can also be accomplished by negative regulation of one repair pathway by another. For example, ATM dependent processing at DNA DSBs that promotes DNA end resection during

HR can be inhibited by the NHEJ factor 53BP1 (Bunting et al. 2010). 53BP1 is thought to encourage NHEJ by stabilizing broken DNA ends and increasing their ability to detect and ligate to one another (Difilippantonio et al. 2008; Dimitrova et al. 2008). In cancer cells with mutations in the breast cancer associated protein BRCA1 which display defects in the resection step of HR, chromosomes are aberrantly joined into complex re-arrangements due to the function of pro-NHEJ factors 53BP1 and LIG4. Depletion of 53BP1 in these cells restores DNA end resection and thus partially restores HR (Cao et al. 2009; Bunting et al. 2010; Bouwman et al. 2010). This suggests that in normal cells BRCA1 can compete with and overcome 53BP1 function at DNA DSBs to allow DNA end resection and promote high fidelity HR mediated repair.

In addition to BRCA1 mutant cells, it has been demonstrated that the genetic instability and sensitivity of FA cells to DNA crosslinking agents is also due to aberrant NHEJ (Adamo et al. 2010; Pace et al. 2010). This could suggest that FA proteins normally promote HR and suppress NHEJ, however not all 16 FA proteins have been examined for this function. Taken together, these observations reveal that in the absence of appropriate repair pathway choice, incorrect pathways can be utilized and cause detrimental outcomes resulting in genomic instability and ultimately cancer.

The DDR and Chromatin

Although the DDR has been well characterized at the DNA level, it is important to consider the DDR within the context of chromatin. Unlike programmed events such as replication of the genome in S phase, the DDR must be elicited at any point in time in response to genomic insult and must overcome the physical barriers that are associated with chromatin structure. Chromatin comprises DNA wrapped around histone proteins and also associates with a variety of non-histone proteins that facilitate the higher order structure organization of the genome allowing it to compact into the nucleus (Kornberg 1977; Probst et al. 2009; Li & Reinberg 2011).

The fundamental unit of chromatin is the nucleosome which contains DNA wrapped around a histone octamer composed of a histone (H3-H4)₂ tetramer surrounded by two histone H2A-H2B dimers (Luger et al. 2012). The presence of linker DNA serves to connect adjacent particles and associates with linker histones (Happel & Doenecke 2009). The composition of nucleosomes in chromatin can vary due to histone posttranslational modifications or the existence of histone variants (Probst et al. 2009). Over the last decade, a collection of studies have demonstrated the importance of these modules whose configuration and spatial organization are a source of information storage for the cell contributing to cellular functions and identity.

DNA repair and signaling machineries must work in concert with chromatin-associated elements in order to access and repair DNA damage. There are two well studied classes of enzymes that facilitate this process, (1) enzymes that modify histones and (2) ATP-dependent chromatin remodeling enzymes. Histone modifying enzymes function by posttranslationally modifying residues within the tail of histones or by modifying histone binding proteins. These modifications include phosphorylation, acetylation, methylation, poly(ADP-ribosylation), ubiquitination, or SUMOylation in addition to other less studied modifications. These modifications can alter the arrangement of nucleosomes, but more typically serve as a signal or code that recruits proteins to chromatin, especially in response to DNA damage (van Attikum & Gasser 2009).

Chromatin remodeling enzymes function to slide nucleosomes along DNA, promote general nucleosome metabolism by exchanging histones or histone dimers, or evict nucleosomes from chromatin altogether (Clapier & Cairns 2009). All chromatin remodeling complexes in the SWI2/SNF2 family contain catalytic subunits with an ATPase/helicase domain. Distinct functional motifs within the catalytic subunit, but outside of the ATPase/helicase domain, determine the classification of chromatin remodelers into four subfamilies including SWI/SNF, ISWI, CHD, and INO80 (Clapier & Cairns 2009). Each subfamily of chromatin remodelers contains specific affinity for different histone or nucleosome modifications. For instance, the SWI/SNF subfamily contains a bromodomain

that binds acetylated histone tail residues. The ISWI subfamily contain HAND, SANT, and SLIDE domains that specifically bind DNA wrapped around nucleosomes. The CHD subfamily contain tandem chromodomains that have affinity for methylated histones and lastly, while the INO80 subfamily does not have a specific domain to bind modified histones, they contain a unique insertion within their ATPase domain (Clapier & Cairns 2009).

I will now highlight some of the cornerstone discoveries and recent findings that have enabled a better understanding of the DDR within the context of chromatin. I will discuss the key roles of histone modifications and chromatin remodeling factors and for clarity focus on the DDR to DNA DSBs.

The first histone modification discovered that was induced by DNA damage was the ATM dependent phosphorylation of the histone variant H2A.X (Rogakou et al. 1998). H2A.X is immediately phosphorylated in response to DNA DSBs and is often used as a nuclear marker of DNA damage. This H2A variant is different between species, but its DNA damage induced phosphorylation, commonly referred to as γ H2A.X, is conserved highlighting the importance of this modification in the DDR. The role of γ H2A.X is primarily to coordinate and spread DDR induced signals. At DNA DSBs, γ H2A.X recruits mediator of DNA damage checkpoint protein 1 (MDC1), which itself is targeted by several protein kinases including ATM (Jungmichel & Stucki 2010). ATM then

promotes spreading of γ H2A.X to neighboring nucleosomes. γ H2A.X can also be induced in response to replication stress. In this situation MDC1 promotes expansion of γ H2A.X by recruiting the adapter protein TOPBP1, which binds and activates ATR at stalled replication forks (Wang et al. 2011). In either event bidirectional spreading of γ H2A.X contributes to checkpoint amplification and defines chromatin regions where the DDR is confined (Yuan et al. 2010). Exactly how this confinement is achieved is still an active area of investigation. One possibility is that some chromatin regions are more accommodating to γ H2A.X, while others, especially condensed heterochromatic regions, are not permissive (Kim et al. 2007). Several phosphatases have been implicated in the dephosphorylation of γ H2A.X, namely PP2AC α , PP2AC β , PP4C, PPC6, and WIP1 in mammals. Depletion of these phosphatases inhibits γ H2A.X removal, DNA DSB repair, and increases cellular sensitivity to irradiation (Keogh et al. 2006; Chowdhury et al. 2008; Douglas et al. 2010; Nakada et al. 2008; Macurek et al. 2010; Cha et al. 2010).

H2A and its variant H2A.X can also be ubiquitinated at DNA DSBs by the E3 ubiquitin ligases RNF8 and RNF168 using a non-proteasomal lysine (K63) based linkage (Kolas et al. 2007; Huen et al. 2007; Mailand et al. 2007; Pinato et al. 2009; Stewart et al. 2009; Doil et al. 2009). This occurs with the help of the E2 ubiquitin conjugating enzyme UBC13 (Kolas et al. 2007; Zhao et al. 2007). The RNF8/RNF168-dependent K63 ubiquitin chains on H2A histones are binding

substrates for RAP80, which forms a complex with BRCA1, BRCC36, ABRAXAS, and MERIT (Wang et al. 2007). RAP80 binding to BRCA1 has been shown to limit CtIP-BRCA1 binding at broken DNA ends, inhibiting DNA end resection and HR (Coleman & Greenberg 2011; Hu et al. 2011). On the contrary, others have shown that the RNF8/RNF168 ubiquitin cascade can promote ubiquitin dependent loading of RAD18 and RAD51C to DSBs to instead promote repair by HR (Huang et al. 2009). Thus, while the RNF8/RNF168 ubiquitin cascade coordinates the ordered recruitment of DNA repair factors to DSBs, how specificity and pathway choice is achieved in this context remains to be determined. The deubiquitinating (DUB) enzyme OTUB1 could provide a layer of specificity in this pathway. Alternative to its DUB cleavage activity, OTUB1 negatively regulates RNF168 mediated K63 ubiquitin linkages by directly binding to the E2 UBC13 preventing the interaction between UBC13/RNF168 and inhibiting ubiquitination (Nakada et al. 2010; Wiener et al. 2012; Sato et al. 2012).

Alternative to ubiquitination, histone methylation at nucleosomes is controlled by histone methyltransferases (HMTs). These enzymes contain catalytic SET domains that transfer methyl groups from S-adenosylmethione, an important methyl donor for many metabolic reactions in the cell, to specific arginine or lysine residues on histones. One important methylation moiety enriched at DNA DSBs is H3K36me₂, which is mounted by the HMT Metnase. H3K36me₂ is required for the accumulation of both sensor proteins NBS1 and

Ku70 at DSBs where it promotes DNA repair by NHEJ (Fnu et al. 2011). Alternatively, the removal of methylation marks from histones can serve to regulate repair at DNA DSBs by pausing transcription and promoting DNA repair. The histone methyl mark H3K4me3 is normally required for transcriptional activation, but is removed in response to DSBs by the demethylase JARID1A (Seiler et al. 2011). This process is thought to promote transcriptional silencing in *cis* at chromatin associated with DNA DSBs to allow DNA repair.

Acetylation is another posttranslational modification that regulates DNA repair in the context of chromatin. Several subunits of the mammalian SWI/SNF remodeling complex and the acetyltransferase TIP60 are recruited to DNA DSBs where they locally decrease nucleosome stability (Xu & Price 2011; Murr et al. 2006). TIP60 directly acetylates ATM, histones H2A/H2A.X, and H4 in response to DNA damage and is required for DNA DSB repair (Gorrini et al. 2007; Murr et al. 2006; Bird et al. 2002; Sun et al. 2005; Sun et al. 2010; Kusch et al. 2004). The enzymatic activity of both SWI/SNF and TIP60 is essential for the downstream steps of DSB repair including histone ubiquitination and the recruitment of mediator proteins BRCA1 and 53BP1 (Xu & Price 2011; Murr et al. 2006).

Lastly, the nucleosome remodeling complex (NuRD) has been shown to function in DNA DSB repair by orchestrating several posttranslational

modifications with chromatin remodeling. The catalytic subunits of the NuRD complex are chromodomain proteins CHD3 and CHD4. These subunits are mutually exclusive and associate with the histone deacetylases HDAC1 and HDAC2 to couple histone deacetylation with chromatin remodeling (Lai & Wade 2011). It is important to note that expression of several NuRD complex components was shown to be reduced in patient cells from the progeria Hutchinson-Gilford syndrome and from naturally aged cells (Pegoraro et al. 2009). Reduced expression of these subunits corresponded with increased γ H2A.X expression, a histone marker of DNA damage described previously, and with loss of heterochromatic structures. These findings suggest that the NuRD complex could prevent the accumulation of DNA damage by maintaining higher order chromatin structures. Furthermore, many NuRD complex members accumulate in foci at sites of DNA damage and several members associate with the central DDR kinase ATR (Chou et al. 2010; Larsen et al. 2010; Smeenk et al. 2010; Polo et al. 2010; Goodarzi et al. 2011).

Interestingly, several studies have shown that NuRD subunits CHD3 and CHD4 have distinct roles in the DDR to DNA DSBs. CHD4 was shown to be targeted by the kinase ATM, but this phosphorylation event was not required to recruit CHD4 to sites of laser induced DNA DSBs (Matsuoka et al. 2007; Polo et al. 2010; Urquhart et al. 2011). CHD4 was also shown to be recruited to DSBs in a manner dependent on PARP activity and was demonstrated to bind PAR

moieties *in vitro* even though it does not contain a PAR binding domain (Polo et al. 2010). CHD4 also interacts with the FHA domain of the ubiquitin ligase RNF8 and has been shown to locally decondense chromatin at DSBs with RNF8 allowing for damage induced ubiquitination by RNF8/RNF168 and downstream recruitment of BRCA1 and 53BP1 (Larsen et al. 2010; Smeenk et al. 2010; Luijsterburg et al. 2012). In stark contrast, CHD3 is thought to function as an inhibitor of DNA DSB repair specifically in heterochromatin. Inhibiting the repressive actions of CHD3 on heterochromatin was shown to facilitate chromatin decondensation and promote a DDR. CHD3 interacts with the constitutively SUMOylated form of the Krüppel-associated box repression (KRAB) domain containing protein KAP-1 (also known as TRIM28). However, in response to DNA damage ATM phosphorylates KAP-1 and this modification disrupts its interaction with CHD3 to induce heterochromatin relaxation (Goodarzi et al. 2008; Goodarzi et al. 2011). Depletion of the heterochromatin factors HDAC1, HDAC2, and HP1 alleviate the requirement for ATM at heterochromatin associated DSB repair (Goodarzi et al. 2008). Heterochromatic phospho-KAP-1 focus formation and subsequent decondensation was also shown to be dependent on MDC1, RNF8, RNF168, and 53BP1. 53BP1 spatially concentrates the MRN sensory complex at late repairing heterochromatic DNA DSBs to enhance ATM activity and presumably allows repair by NHEJ (Noon et al. 2010).

It is clear from the above overview that a complex arrangement of histone modifications and remodeling of chromatin is required for proficient DNA damage induced signaling and repair (Figure 1-1, general model). Some complexes have been implicated in specific repair events or are required for the subsequent accumulation of additional repair factors. The specificity of these factors for histone modifications and function in altering nucleosome occupancy at DNA surrounding DSBs still remains an active area of investigation. Future studies using multidisciplinary approaches combining quantitative proteomics and microscopy techniques could enable a better understanding of chromatin dynamics in response to DNA damage and determine the interdependency of different remodeling complexes.

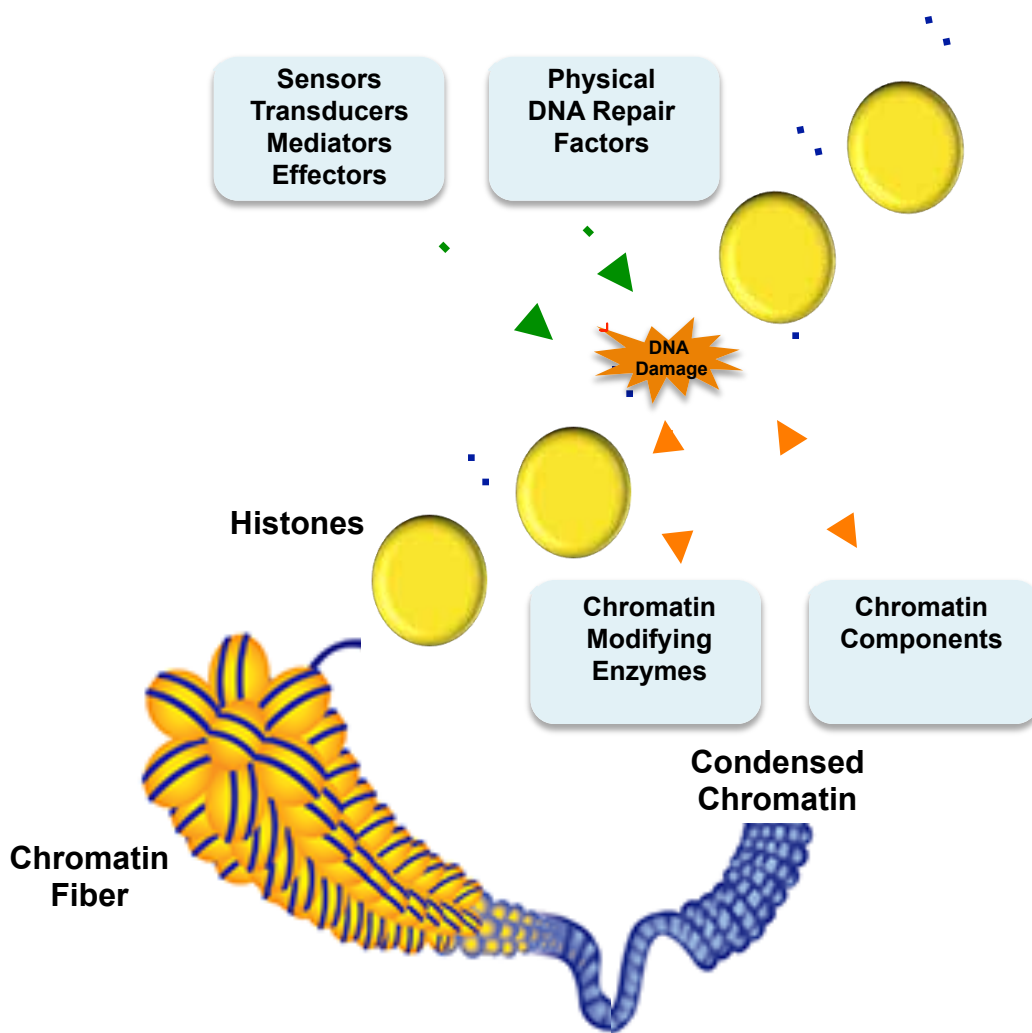


Figure 1-1 General schematic of the DNA damage response in the context of chromatin. The dynamics of proteins involved in the of DNA damage response are depicted. DNA damage checkpoint and repair factors are recruited (green arrows), while modulators of chromatin organization and general proteins that compose chromatin operate in both directions (Orange arrows).

Loss of Function in the DDR, Cancer Development, and Targeted Therapies

Highlighted in the first section of this introduction is the characteristic that human tumors are genetically unstable (Hanahan & Weinberg 2011). Inactivation of DDR genes enhances the accumulation of mutations and DNA aberrations that promote transformation and cancer development. This is evident based on the cancer-prone phenotypes of several DDR syndromes. I will describe some of these syndromes as well as the potential therapeutic avenues that could be exploited due to inherent DNA repair defects of these cells.

Lynch syndrome, also known as hereditary non-polyposis colon cancer (HNPCC), results from inherited heterozygous mutations in the DNA MMR genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* (Spry et al. 2007). Mutations in these genes can also predispose the development of cancers in the endometrium, ovary, stomach, and kidneys (Lynch et al. 2009). One form of genomic instability associated with HNPCC is the expansion or contraction of repetitive microsatellite DNA sequences termed microsatellite instability (MSI) (Lynch et al. 2009). However, some HNPCC-associated mutations have unknown pathogenicity and do not present with MSI. Our laboratory recently uncovered one such example by investigating an *MLH1* clinical mutation with a leucine (L)-to-histidine (H) amino acid change at position 607 that ablated *MLH1* binding to the Fanconi anemia-associated protein *FANCD1* (Xie et al. 2010). Instead of directly promoting MSI, loss of *MLH1* binding to *FANCD1* altered MMR signaling in

response to DNA damaging. Importantly, loss of MLH1 binding to FANCD1 provided insight towards directed therapy as loss of the interaction uniquely sensitized cells to DNA cross-linking chemotherapies (Xie et al. 2010). Other therapies have exploited defects arising from MMR deficiency rather than exploiting defects arising from specific mutations in MMR proteins. The inhibitor of DNA base synthesis, methotrexate, has been demonstrated to uniquely kill MMR-deficient cells by promoting the accumulation of oxidative lesions (Martin et al. 2010). Furthermore, depletion studies showed that loss of the BER polymerase Pol β or the mitochondrial polymerase Pol γ resulted in synthetic lethality in combination with MSH2 and MLH1 deficient tumor cells, again owing to an increased frequency of oxidative lesions (Martin et al. 2010). Further assessment of these therapeutic avenues will be important, especially given that many sporadic forms of colon cancer also contain loss of function mutations in MMR genes (Liu et al. 1995).

Familial forms of breast, ovarian, and pancreatic cancer are also linked to defects in HR pathway associated genes such as *BRCA1*, *BRCA2*, *FANCD1/BACH1*, *PALB2*, and *RAD51C* (Miki et al. 1994; Wooster et al. 1995; Walsh & King 2007; Levy-Lahad 2010; Cantor & Guillemette 2011). Patients with *BRCA2* mutations also show increased incidence of male breast, pancreatic, and prostate cancer (Moynahan & Jasin 2010). Additionally, inactivation of the ATM dependent signaling axis in cancer has become apparent as mutations in *CHK2*,

ATM, *NBS1*, and *RAD50* are all associated with a 2-fold increased risk of developing breast cancer (Walsh & King 2007). In patients with *BRCA1* or *BRCA2* mutant tumors the therapeutic use of PARP inhibitors has been an active area of investigation (Jackson & Bartek 2009). Screening of chemical libraries and the chemical refinement of compounds identified have already led to the third generation of PARP inhibitors currently in use in clinical trials for BRCA patients (Rouleau et al. 2010; Lord & Ashworth 2012). The synthetic lethality of PARP inhibition in patients with BRCA mutations has been proposed to result from the accumulation of unrepaired DNA breaks or from the inhibition of PARP release from a DNA lesion (Rouleau et al. 2010). In either event, both types of DNA lesions would contribute to the collapse or stalling of replication forks, which could increase the number of DNA DSBs in the genome. In normal *BRCA1/2* wild-type cells the effect of PARP inhibition is buffered by active homologous recombination, which repairs DNA DSBs.

The strong link between familial breast and ovarian cancer with FA syndrome has also become apparent (Levy-Lahad 2010). Mono-allelic inheritance of mutations in *BRCA2*, *FANCI/BACH1*, and *PALB2* predispose to breast and ovarian cancer as described previously, however, bi-allelic inheritance of these genes results in FA syndrome within the complementation groups FA-D1, FA-J, and FA-N, respectively. Most recently, bi-allelic mutations in *RAD51C* were also identified in an FA-like disorder (Levy-Lahad 2010; Vaz et al. 2010).

Not surprisingly, FA cells have underlying genomic instabilities showing spontaneous and DNA crosslinker induced chromosome fragility, a hallmark that is important for diagnosis in patients. FA patients are also associated with an increased risk of pre-leukemic syndromes and cancer including myelodysplasia (MDS) and acute myeloid leukemia (AML) (Bagby & Meyers 2007; Dokal & Vulliamy 2008; Quentin et al. 2011). FA patients typically undergo progressive bone marrow failure (BMF) during childhood, which requires allogeneic hematopoietic stem cell (HSC) transplant (Gluckman & Wagner 2008; Shimamura & Alter 2010). Most attempts to uncover the mechanisms leading to BMF in FA patients have been unsuccessful due to difficulties associated with studying a rare human disease with low bone marrow cells and because the mouse model of FA does not fully recapitulate the phenotypes of human FA (Parmar et al. 2009). Recent work analyzing a large series of primary bone marrow samples from FA patients in functional models revealed that hematopoietic stem and progenitor cells (HSPCs) from these patients are impaired due to exacerbated p53/p21 activation and G0/G1 cell cycle arrest activated by the intrinsic DNA repair defect (Ceccaldi et al. 2012). Depletion of p53 in this study rescued the HSPC defects and clonogenic ability in several models, which implies that targeting p53 could have therapeutic potential to prevent BMF in FA patients. However, while the direct targeting of p53 with small molecule inhibitors could likely enhance HSPC progression and mitigate

BMF, this approach would also likely enhance genomic instability and increase the risk of clonal evolution encouraging malignancy (Gudkov & Komarova 2010). Perhaps a better approach to ameliorate BMF in FA patients could come from the use of new drugs that inhibit pro-inflammatory cytokines or oxidative stress as the induction of p53/p21 can arise from excess cellular stress or from unresolved DNA damage (Dufour & Svahn 2008).

In summary, the proper coordination and fidelity of DNA repair processes is essential for development and for suppressing cellular transformation. Defects in the DDR not only predispose to certain types of cancer as described in this section (Table 1-1), but can also alter the sensitivity of tumor cells to therapies that target specific aspects of the DDR.

Disease	Gene(s) Mutated	DDR Defect	Cancer Type
Hereditary NonPolyposis Colorectal Cancer (HNPCC) also called Lynch Syndrome	<i>MSH2</i> , <i>MSH3</i> , <i>MSH6</i> , <i>MLH1</i> , <i>PMS2</i>	DNA Mismatch repair	Colorectal, Ovarian, Endometrial cancer
Familial Breast/Ovarian cancer	<i>ATM</i> , <i>BRCA1</i> , <i>BRCA2</i> , <i>FANCI</i> , <i>CHK2</i> , <i>NBS1</i> , <i>PALB2</i> , <i>RAD50</i> , <i>RAD51C</i>	Homologous recombination, DNA Damage Signaling	Breast cancer, Ovarian cancer
Fanconi anemia (FA)	<i>FANCA-C</i> , <i>FANCD1</i> , <i>D2</i> , <i>FANCE-G</i> , <i>FANCI</i> , <i>J</i> , <i>L-Q</i>	Interstrand crosslink repair, Homologous recombination	Acute myeloid leukemia, Myelodysplasia, Squamous cell carcinoma

Table 1-1 Human genetic diseases associated with defects in DNA damage response (DDR) genes and associated cancer types.

Concluding Remarks

To combat sources of DNA damage cells have evolved many complex pathways collectively known as the DDR. The DDR consists of signal transduction pathways that sense DNA damage and mount tightly coordinated responses to pause the cell cycle and repair DNA. An arsenal of different enzymes specific for the type of DNA damage induced is employed in concert with enzymes that modify histones and ATP-dependent enzymes that remodel chromatin to access the damage. The inactivation of genes associated with the DDR causes the accumulation of mutations and DNA aberrations that promote transformation and cancer development. As a result, one common characteristic for all human tumors is genetic instability.

For my first thesis investigation, I chose to characterize the function of the breast/ovarian cancer tumor suppressor and Fanconi Anemia associated protein FANCD1, in a role that has not previously been implicated for this protein, the DDR to ultraviolet (UV) irradiation. FANCD1 is best known for its function with BRCA1 in response to DNA double strand breaks and for its function in DNA interstrand crosslink repair with the FA pathway (Cantor et al. 2001; Litman et al. 2005). I found that FANCD1 is recruited to sites of UV induced DNA damage in a manner dependent on MMR factors and dual incision by NER endonucleases XPD and XPG in S-phase of the cell cycle. Surprisingly, BRCA1 binding was not required for FANCD1 localization to sites of damage. In response to UV irradiation FANCD1

supports checkpoint induction, the arrest of DNA synthesis, and suppresses UV induced point mutations. Despite these findings I noted that FANCD1 deficient cells were not sensitive to UV induced DNA damage and thus considered if FANCD1 loss could be associated with tumorigenesis in skin cells. In sequenced melanomas I found somatic mutations in FANCD1 that were previously associated with hereditary breast cancer and cancer prone FA.

In the second part of my thesis research I investigate the factors that contribute to chemo-resistance in BRCA2 mutant ovarian cancers. As described in the second part of my introduction BRCA2 mutant tumors have defects in DNA repair by HR and are thus initially sensitive to DNA damage inducing chemotherapies. However, ovarian tumors are typically asymptomatic and diagnosed at more advanced stages, thus therapeutic resistance eventually develops. One mechanism of resistance includes reversion mutations in BRCA2 that restore functional protein and RAD51-based HR (Sakai et al. 2008; Sakai et al. 2009; Edwards et al. 2008). It is unknown how chemo-resistance develops without reversion mutations. Here, I found in a genome-wide shRNA screen, that depletion of the chromatin-remodeling factor CHD4 confers cisplatin resistance in BRCA2 mutant cells. Rescue by CHD4 depletion does not improve RAD51 foci formation and occurs through a novel mechanism. Consistent with CHD4 modulating therapeutic response, I discovered that low CHD4 expression

correlates with poor response and shortened overall survival in patients with *BRCA2*-mutant ovarian tumors.

These two studies help to better understand the roles of DDR pathways and chromatin remodeling factors in the suppression of UV-induced mutations and treatment of ovarian tumors with chemotherapy, respectively. Both discoveries contribute to our understanding of the DDR and its relationship with hereditary cancers.

CHAPTER II:

FANCI FUNCTION IN THE DNA DAMAGE RESPONSE TO UV IRRADIATION

Preface

This research chapter derives from work I started in my first year in Sharon Cantor's lab in February of 2008 and lasted until December of 2013 when it was published in the journal *Cancer Research* entitled "FANCI Localization by Mismatch Repair is Vital to Maintain Genomic Integrity after UV Irradiation". I am the first author of this publication with additional authors Amy Branagan, Min Peng, Aashana Dhruva, Orlando Scharer, and Sharon Cantor.

This work originally stemmed from work I completed to help former lab members Jenny Xie, Rachel Litman, and Shu Wang produce the *Oncogene* publication "Targeting the FANCI-BRCA1 Interaction Promotes a Switch from Recombination to Pol Eta-Dependent Bypass" for which I am third author. During that time Min and myself recognized that FANCI accumulated in nuclear foci in response to UV irradiation. I also performed mutation frequency assays in cells in response to various DNA damaging agents and found that loss of FANCI significantly elevated the frequency of mutations in response to UV irradiation. Together, these findings ultimately set me on the path to functionally investigate the role of FANCI in the DDR to UV irradiation.

In this data chapter, I performed all the technical experiments with the help of Min Peng retrovirally complementing the patient derived FA-J cells with various FANCI mutant species. Shu Wang performed the GFP-Pol Eta foci experiments, Amy Branagan performed the FACS based RPA phosphorylation assay analyzing the UV induced checkpoint, and Aashana Dhruva aided in lysate preparation and running SDS-PAGE gels for experiments depleting MMR proteins. Microscopy experiments were analyzed double blind with the essential help of many undergraduates and high school students who volunteered over the course of several summers with a special internship program through Bancroft High School. The model drawn in Figure 2-28 was initially designed by graphic artist Miroslav Koulis hired by Sharon Cantor, but the many alterations and modifications to this drawing achieved in the final model were done by myself.

Abstract

Nucleotide excision repair (NER) is critical for the repair of DNA lesions induced by ultraviolet (UV) radiation, but its contribution in replicating cells is less clear. Here, we show that dual incision by NER endonucleases including XPF and XPG promotes the S-phase accumulation of the BRCA1 and Fanconi anemia (FA)-associated DNA helicase FANCD1 to sites of UV-induced damage. At these sites, FANCD1 promotes RPA phosphorylation and the arrest of DNA synthesis following UV irradiation. Interaction defective mutants of FANCD1 reveal that BRCA1 binding is not required for FANCD1 localization, whereas interaction with the mismatch repair (MMR) protein MLH1 is essential. Correspondingly, we find that FANCD1, its direct interaction with MLH1, and the MMR protein MSH2 function in a common pathway to ensure NER-dependent and independent S-phase checkpoints. Further supporting an important role for FANCD1 in the response to UV irradiation, FANCD1 suppresses UV induced point mutations. In combination with the fact that FANCD1 deficient cells are not sensitive to killing by UV light, we considered that FANCD1 loss could be associated with tumorigenesis. Along these lines, in melanoma we found somatic mutations in FANCD1 expected to inactivate the ATPase/helicase activity that were previously associated with hereditary breast cancer and FA. Thus, we propose collaborations between FA, NER and MMR are necessary to initiate checkpoint activation in replicating human cells to limit genomic instability.

Introduction

Repair of ultraviolet (UV) irradiation-induced DNA damage depends on the nucleotide excision repair (NER) pathway. Underscoring the essential role of NER in repair of UV-induced DNA damage, inherited defects in NER genes result in the skin cancer-prone disease Xeroderma pigmentosum (Cleaver 1968). In non-replicating cells NER factors sense UV-induced DNA damage and excise the lesion in a multi-step process. The remaining short single-stranded DNA (ssDNA) region serves as a template for repair synthesis, “gap” repair (Hoeijmakers 2001; Gillet & Schärer 2006). Lesions escaping NER gap repair stall replication forks and initiate checkpoint responses. Some NER factors interact with the replisome and contribute to the early S phase checkpoint response (Bomgarden et al. 2006; Gilljam et al. 2012). In post-replication repair lesions are managed largely through DNA-damage tolerance mechanisms (Sogo et al. 2002; Byun et al. 2005; Cortez 2005). Among the recent factors involved in this process is the hereditary breast cancer-associated gene product BRCA1, which function independently of NER to suppress mutations (Pathania et al. 2011).

Several lines of evidence indicate that UV-induced damage is also limited by proteins of the DNA mismatch repair (MMR) pathway. MMR factors induce checkpoints, apoptosis, preserve genomic stability, and suppress cancer induced by UV irradiation (Nara et al. 2001; Meira et al. 2002; Yoshino et al. 2002; Seifert et al. 2008; Borgdorff et al. 2006). The mechanism by which MMR functions in

response to UV irradiation could stem from its general role in genome surveillance and mismatch correction. Canonical MMR begins with the recognition of replication errors where MSH2-MSH6 (MutS α) or MSH2-MSH3 (MutS β) assemble and recruit the heterodimer MLH1-PMS2 (MutL α) (Kunkel & Erie 2005). These complexes function in the repair of mismatched bases. As such, loss of MMR confers a mutator phenotype and a predisposition to hereditary nonpolyposis colon cancer (HNPCC) (Jiricny 2006). However, it is also well appreciated that MMR proteins respond to DNA damage from exogenous sources, such as to DNA alkylating agents, known to induce mismatches following DNA replication (Kaina et al. 2007). In response to UV irradiation, MMR factors could have an alternative non-canonical role in UV lesion processing given that the MSH2-MSH6 complex directly binds UV lesions (Mu et al. 1997; Wang et al. 1999). Clarifying how MMR contributes to genomic stability in the UV response will be central to understanding the HNPCC variant, Muir-Torre syndrome that is characterized by skin cancers (Mathiak et al. 2002; Kruse et al. 1998; Suspiro et al. 1998).

Both the MMR protein, MLH1 and BRCA1 bind directly to the DNA helicase FANCD1 that hitherto has not been described as a factor in the UV response, but has essential functions in activating checkpoints following replication stress (Cantor et al. 2001; Peng et al. 2007; Gong et al. 2010; Cotta-Ramusino et al. 2011; Xie et al. 2012). FANCD1 is mutated in hereditary breast

and ovarian cancer as well as in the rare cancer-prone syndrome Fanconi anemia (FA) (Cantor et al. 2001; Litman et al. 2005). Complementation studies using FANCD1 deficient (FA-J) patient cells demonstrated that MLH1 binding is critical for FANCD1 function in the repair of DNA interstrand crosslinks (ICLs) (Peng et al. 2007). Here, we reveal that MLH1 binding to FANCD1 is also essential for the response to UV-induced damage, in which FANCD1 promotes an S phase checkpoint point and limits UV-induced mutations. Because dual incision by NER also promotes FANCD1 accumulation at sites of UV induced damage, and the NER endonuclease XPF was recently shown to be an FA gene similar to FANCD1, our analysis suggests that FA, MMR and NER pathways collaborate to process UV lesions in S phase cells to preserve the genome (Bogliolo et al. 2013; Kashiwaga et al. 2013).

Results

FANCI accumulation at sites of UV induced damage is dependent on NER dual incision

We examined the response of FANCI to UV irradiation by assessing whether FANCI accumulated at sites of UV-induced damage. Following UV-irradiation through 3 or 5 micron filters to generate sites of localized UV damage (LUDs) (Moné et al. 2001; Volker et al. 2001), we found that FANCI co-localized with UV-induced 6-4 pyrimidine-pyrimidones (6-4 PPs), cyclobutane pyrimidine dimers (CPDs), and the NER endonuclease XPF in the breast cancer cell line, MCF7. FANCI localization to 6-4 PP- or XPF-positive LUDs peaked ~3 h after UV irradiation and diminished by ~12 h (Figure 2-1).

To address the relationship of FANCI to NER, we used *XP* cell lines and their functionally complemented counterparts. We found that the accumulation of FANCI at LUDs was reduced ~2- to 3-fold in NER-deficient *XP-A*, *XP-F* and *XP-G* cells when compared to wild-type complemented cells (Figures 2-2, 2-3, 2-4). Contributing to FANCI localization was XPF and XPG endonuclease activity as complementation with XPF or XPG nuclease-defective mutant species, XPF^{D676A} or XPG^{E791A} failed to restore robust FANCI accumulation at LUDs between ~1-5h post UV-induced damage (Figures 2-3, 2-4) (Staresincic et al. 2009). Following global UV irradiation, FANCI foci were also more prominent in *XP-F* cells

complemented with wild type XPF endonuclease (Figure 2-5). By contrast, FANCI depletion did not affect the localization dynamics of NER factors (Figure 2-6). Collectively, these data indicate that NER incision events potentiate the accumulation of FANCI to sites of UV-induced damage.

NER promotes the accumulation of FANCI at UV induced damage in S phase

Next, we investigated whether NER contributed to FANCI accumulation at LUDs in a specific cell cycle phase. Cells within S-phase and non-S phase can be easily distinguished after local UV irradiation by staining for 5-ethynyl-2'-deoxyuridine (EdU) incorporation into genomic DNA (Limsirichaikul et al. 2009). In S-phase cells, EdU staining is bright and pan nuclear. In non-S phase cells, EdU staining is restricted to sites of LUDs, representing sites of unscheduled DNA synthesis that occurs during gap repair in NER (Volker et al. 2001; Limsirichaikul et al. 2009; Sertic et al. 2011). Following localized UV irradiation, cells were incubated in media with EdU for 3 h and immunostained with FANCI antibodies. Consistent with a role for XPF in NER dependent gap filling, EdU positive LUDs in non-S phase cells were only present in XPF^{WT} cells (Figure 2-7). FANCI recruitment to LUDs was not significantly improved in non-S phase XPF^{WT} cells, however it was significantly enhanced in S-phase XPF^{WT} cells indicating that XPF potentiates FANCI accumulation in cells undergoing DNA synthesis (Figure 2-7).

FANCD1 localization to sites of UV induced damage is MMR dependent

FANCD1 directly binds BRCA1, which functions in the response to UV irradiation selectively in S/G2 phase cells (Pathania et al. 2011; Cantor et al. 2001). FANCD1 also directly binds MLH1, which along with other MMR factors function in the response to UV irradiation and preserve genomic integrity (Peng et al. 2007; Young et al. 2003). Because both BRCA1 and MLH1 contribute to FANCD1 localization and function in the DNA damage response, we investigated whether BRCA1 or MLH1 interactions were required for FANCD1 localization to LUDs. We analyzed FANCD1 recruitment in FANCD1 deficient *FA-J* patient cells complemented with empty vector, FANCD1^{WT}, the BRCA1-interaction defective mutant (FANCD1^{S990A}), or the MLH1-interaction defective mutant (FANCD1^{K141/142A}) (Peng et al. 2007; Yu et al. 2003). While the FANCD1 species expressed at similar levels, we found that FANCD1^{K141/142A} localization was dramatically reduced as compared to FANCD1^{S990A}, which localized to LUDs just as efficiently as FANCD1^{WT} (Figure 2-8). Importantly, FANCD1 positive LUDs were not detected in FANCD1-null *FA-J* cells unless complemented with wild-type FANCD1 confirming the specificity of our FANCD1 antibody (Figure 2-8).

Further validating that FANCD1 localization to LUDs requires functional MMR, we found that as compared to a non-silencing control (NSC), FANCD1 recruitment to LUDs was severely reduced in U2OS cells depleted of MLH1,

MSH2, or MSH6 (Figures 2-9, 2-10). In contrast, XPC and ERCC1 recruitment to LUDs was not affected by MSH2 depletion (Figure 2-11), indicating that MMR is required for accumulation of FANCI at LUDs, but not XPC or ERCC1. Likewise, MSH2 recruitment to LUDs was similar in vector and XPF^{WT} complemented *XP-F* cells while as expected ERCC1 was only present in the XPF^{WT} complemented *XP-F* cells suggesting that MMR and NER accumulation at LUDs is not inter-dependent (Figure 2-11). We also noted that the residual accumulation of FANCI found at LUDs in *XP-F* cells was eliminated by depletion of MSH2 (Figure 2-12), suggesting that NER and MMR operate in a parallel manner to support FANCI localization. In the *XP-F* cells, however MSH2 depletion did not perturb FANCI nuclear or chromatin localization suggesting MMR and NER contribute to FANCI localization to LUDs as opposed to nuclear import (Figure 2-13).

We expected that both NER and MMR would also be present in S phase cells given that they contribute to the S phase localization of FANCI. However, NER proteins are best known for their UV repair function in non-S phase cells and from the literature it was not clear if MMR proteins had a cell cycle dependent localization to LUDs (Gillet & Schärer 2006). We used primary immortalized 48BR fibroblasts that have been used to characterize NER proteins in gap repair by means of EdU incorporation (Sertic et al. 2011). While XPF was clearly present in non-S phase cells at sites of gap filling, as expected, we also detected XPF in nearly all LUDs in S phase cells, ~95% (Figure 2-14). MLH1

and MSH2 were also present at LUDs with a similar percent in both non-S and S phase cells. Instead, FANCI was primarily at LUDs in S phase cells, ~86% and only in ~19% of non-S phase cells (Figure 2-14). Collectively, these studies show that MMR and NER proteins localize to LUDs in both non-S and S phase cells whereas FANCI localizes primarily in S phase cells.

FANCI promotes the UV induced arrest of DNA synthesis and the induction of RPA phosphorylation

UV irradiation activates checkpoint responses and inhibits DNA replication in S phase cells (Kaufmann & Cleaver 1981; Kaufmann 2010). Given the role of FANCI in checkpoint responses and the accumulation of FANCI at LUDs during S-phase, we tested if FANCI contributed to the UV-induced checkpoint response (Kumaraswamy & Shiekhataar 2007; Gong et al. 2010; Xie et al. 2012). By pulsing cells with EdU, we found that *FA-J* cells expressing FANCI^{WT} underwent a 10.5-fold reduction in S-phase cells when examined 16 h after UV irradiation. By comparison, *FA-J* cells expressing vector underwent a 2.0-fold reduction (Figure 2-15), indicating that FANCI contributes to the arrest of DNA synthesis in response to global UV irradiation.

The UV induced arrest of DNA synthesis is also associated with changes in phosphorylation of the single-stranded DNA-binding protein RPA (Carty et al. 1994). Following UV-irradiation, the 32 kDa subunit of RPA is phosphorylated on

several serine residues in the N-terminal of the protein in a cell cycle dependent manner by DNA-PK and cyclin dependent kinases (Niu 1997; Zernik-kobak et al. 1997). By examining phosphorylation of serines4/8 on RPA32 with a specific antibody, we found that in response to global UV irradiation FANCD1 complementation was sufficient to enhance RPA serines4/8 phosphorylation in FA-J cells by fluorescent activated cell sorting (FACS) (Figure 2-16). By FACS analysis basal phospho-S4/8 RPA32 was ~1-2% in both untreated vector and wild-type FANCD1 complemented FA-J cells. Following UV-irradiation, phospho-S4/8 RPA32 was induced to ~22% in FANCD1^{WT} FA-J cells as compared to only ~7% in vector FA-J cells (Figure 2-16). Likewise, using phospho-S4/8 RPA32 immunostaining in conjunction with EdU pulse, we uncovered that phospho- S4/8 RPA32 staining was detected only in S phase cells (Figure 2-17). Furthermore, we found that FA-J patient cells complemented with FANCD1^{WT} or the BRCA1-interaction defective mutant (FANCD1^{S990A}) had significantly greater EdU positive S-phase cells with phospho-S4/8 RPA32 positive LUDs as compared to the FA-J cells complemented with empty vector or the MLH1-interaction defective mutant (FANCD1^{K141/142A}) (Figure 2-17). This finding further suggested that FANCD1 and the FANCD1-MLH1 interaction, but not the BRCA1-interaction contributes to checkpoint responses in S phase cells.

Depletion experiments confirmed the role of FANCD1 in promoting checkpoint responses to UV irradiation. In FANCD1-depleted MCF7 cells,

phospho-S4/8 RPA32 as well as the soluble checkpoint factor phospho-317 CHK1 was reduced compared to non-silencing control (NSC) while total CHK1 and RPA levels were unchanged (Figure 2-18). Co-immunostaining with phospho-S4/8 RPA32 and 6-4 PP antibody to visually mark UV induced LUDs also revealed that phospho-S4/8 RPA32 was significantly reduced in FANCD1 depleted MCF7 cells as compared to NSC (Figure 2-19). Interestingly, by 12h post-UV damage, a small, but significant persistence of UV induced 6-4 PP LUDs remained in FANCD1 depleted cells (Figure 2-19). FANCD1 or MSH2 depletion also consistently enhanced the persistence of 6-4 PP positive LUDs in the male lung cancer cell line, A549 in which the formation of phospho-S4/8 RPA32-positive LUDs was also significantly reduced (Figure 2-20). Furthermore, the combination of FANCD1 and MSH2 depletion was not additive (Figure 2-20), suggesting that FANCD1 and MSH2 function in a common pathway that is not cell type specific.

Recently NER factors were shown to promote the S phase checkpoint response, including RPA phosphorylation in response to UV irradiation (Bomgardner et al. 2006; Auclair et al. 2008; Gilljam et al. 2012). Given that the mechanism by which NER promotes the S phase checkpoint is unclear, we considered whether the NER-dependent accumulation of FANCD1 at LUDs in S phase was required. As before, *XP-F* patient cells were segregated into non-S and S phase cells by labeling with EdU and phospho-S4/8 RPA32 staining was

detected only in S phase cells (Figure 2-21). We found that phospho-S4/8 RPA32 induction was greatest in *XP-F* cells complemented with XPF^{WT} (Figure 2-21). Strikingly, depletion of FANCD1 or MSH2 profoundly reduced the phospho-S4/8 RPA32 induction of XPF^{WT} complemented *XP-F* cells (Figure 2-21). Notably, the residual phospho-S4/8 RPA32 positive LUDs found in vector complemented *XP-F* cells were also reduced by depletion of FANCD1 or MSH2 (Figure 2-21). Thus, FANCD1 promotes S phase checkpoint responses in not only cancer cell lines, but also in non-transformed fibroblasts. Together, these data suggest that MSH2 and FANCD1 contribute to NER-dependent and independent UV-induced phospho-S4/8 RPA32 induction at LUDs in S phase cells. In contrast, when either FANCD1 or MSH2 were depleted, we found gap filling was proficient in the *XP-F* cells complemented with XPF^{WT} (Figure 2-22). Gap filling was also proficient in 48BR cells depleted of FANCD1, MLH1, or MSH2, but reduced in cells depleted of XPF (Figure 2-23). Msh2^{-/-} and Msh2^{+/+} mouse embryonic fibroblasts also had similar levels of gap filling (Figure 2-23), suggesting that FANCD1 and MMR factors are not required for NER-dependent gap filling.

Collectively, our data indicate that FANCD1 contributes to the UV induced arrest of DNA synthesis by potentiating checkpoint induction pathways. While FANCD1 does not contribute to NER-dependent gap repair, it influences the clearance of UV induced lesions in a common pathway with MSH2.

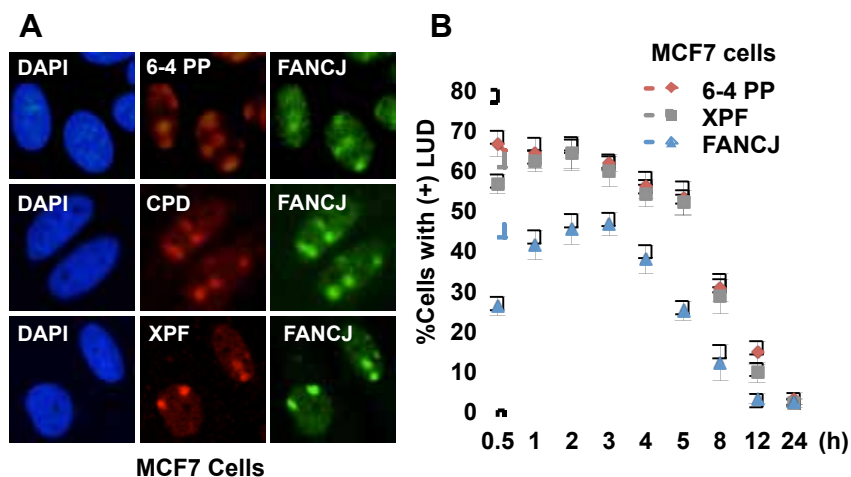
FANCI suppresses UV-induced mutations

Given that FANCI is dispensable for survival after UV exposure, we sought to examine if FANCI preserves the integrity of the genome, as has been found for MMR (Borgdorff et al. 2006; J Xie et al. 2010). Male A549 cells are useful for analyzing mutations at the endogenous hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) locus located on the X-chromosome (Chiu et al. 2006). Similar to other cell lines examined, in A549 cells FANCI localized to sites of UV induced damage as demonstrated by co-precipitation of FANCI with CPD and modified PCNA following UV-induced damage (Figure 2-24).

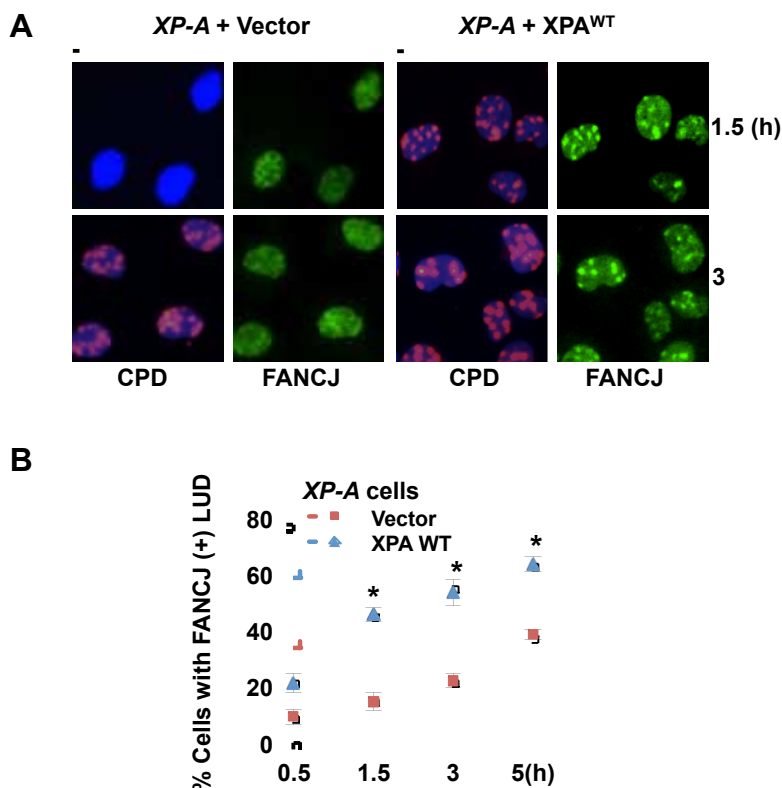
Using RNAi-mediated FANCI silencing, we confirmed that FANCI was not essential for survival following UV irradiation, but was essential for survival following exposure to the DNA cross-linking agent cisplatin (Figures 2-25, 2-26). As compared to NSC, we found that FANCI depletion enhanced UV-induced *HPRT* inactivating mutations determined by clonal selection in 6-thioguanine (Chiu et al. 2006). FANCI depletion did not affect spontaneous *HPRT* mutations, but the frequency of inactivating *HPRT* mutations after 10J/m² UV irradiation was enhanced ~10-fold in A549 cells (Figure 2-26). Sequencing of clones arising from *HPRT* inactivation indicated no gross deletions or rearrangements as a consequence of FANCI deficiency in response to global UV irradiation. Instead, *HPRT* inactivation was predominated by ~8-fold more C to T transitions in both the transcribed strand (TS) and non-transcribed strand (NTS) of *HPRT* in FANCI

depleted cells (Figure 2-26, Table 2-1). These findings suggest that FANCD1 is involved in a specific process that suppresses the formation of point mutations in response to UV irradiation. Further supporting that FANCD1 suppresses inactivating mutations at the *HPRT* locus, FANCD1^{WT}-complementation in FANCD1 deficient FA-J patient cells was sufficient to reduce survival in 6-thioguanine (6-TG) after UV irradiation, indicating that it averted the occurrence of mutations (Figure 2-27). Furthermore, complementation with FANCD1^{WT} enhanced resistance to DNA cross-linking agent, mitomycin C (MMC), while in accordance with the results found in A549 cells, the resistance to UV irradiation was unchanged (Figure 2-27). Together, these results suggest that FANCD1, similar to MMR, contributes to the prevention of mutations in response to UV irradiation, without affecting long-term survival following this treatment (Nara et al. 2001; Meira et al. 2002; Yoshino et al. 2002; Seifert et al. 2008). Thus, we propose that in collaboration with NER, the MMR-FANCD1 pathway is important for the response to UV irradiation in S phase to ensure checkpoint responses, genome stability and limit tumorigenesis (Figure 2-28).

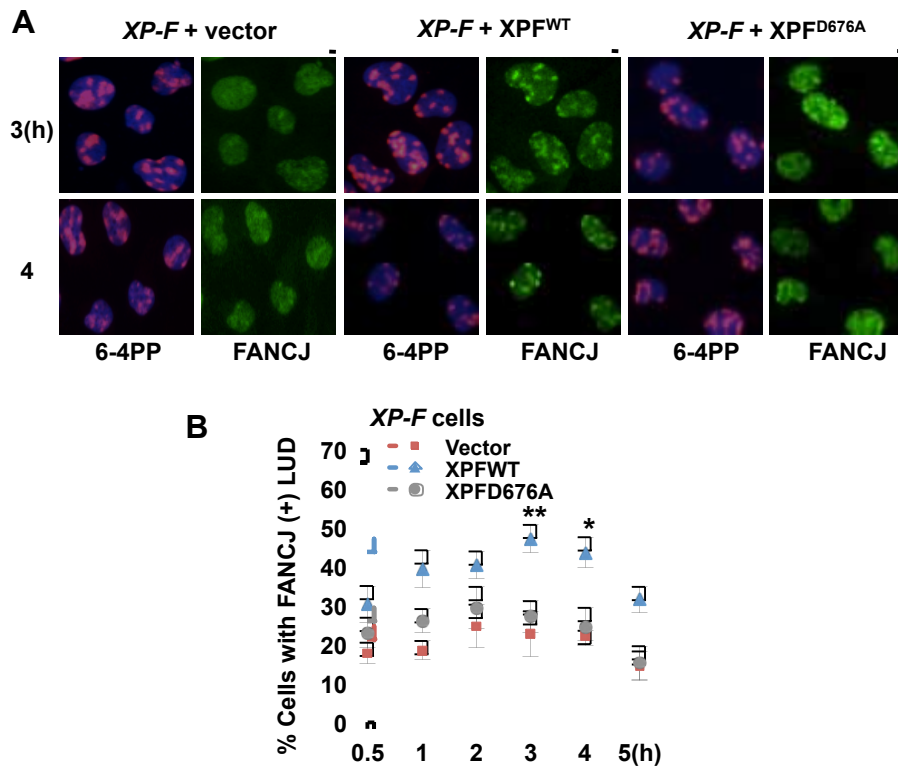
Figures



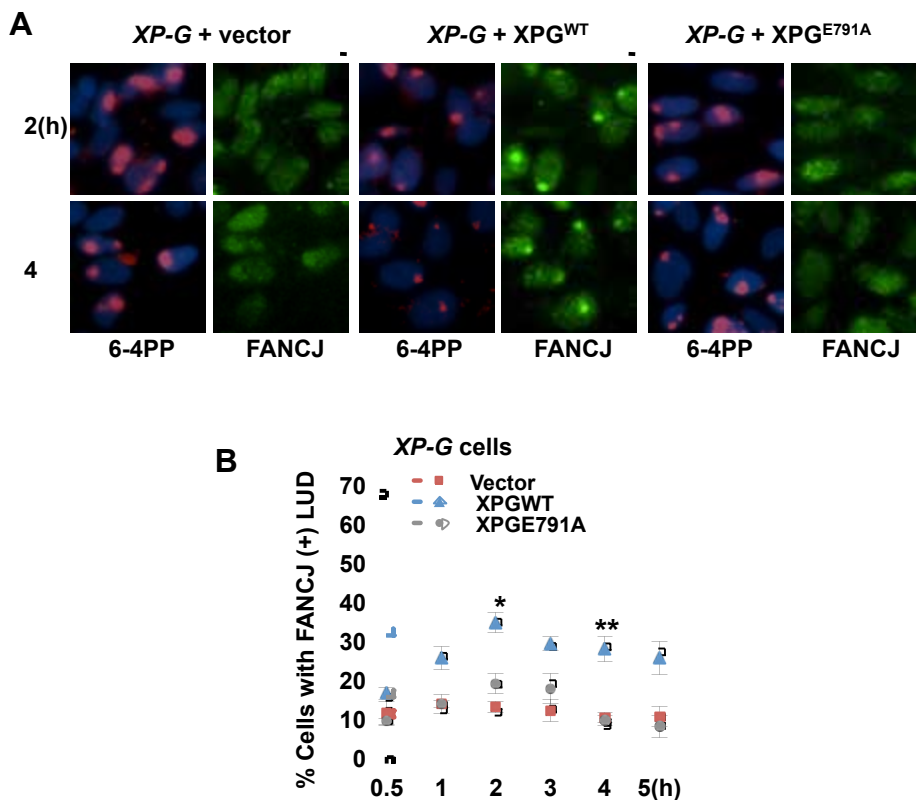
2-1 FANCJ recruitment to sites of local UV-induced damage (LUDs) (A) MCF7 cells were UV irradiated through 5 μ m filters to generate LUDs and co-immunostained with the indicated Abs. Representative images are shown 1 h after UV irradiation. (B) Quantification of MCF7 cells positive for FANCJ, XPF, or 6-4 PP LUDs normalized to DAPI. Error bars represent the standard deviation of the mean of three independent experiments,



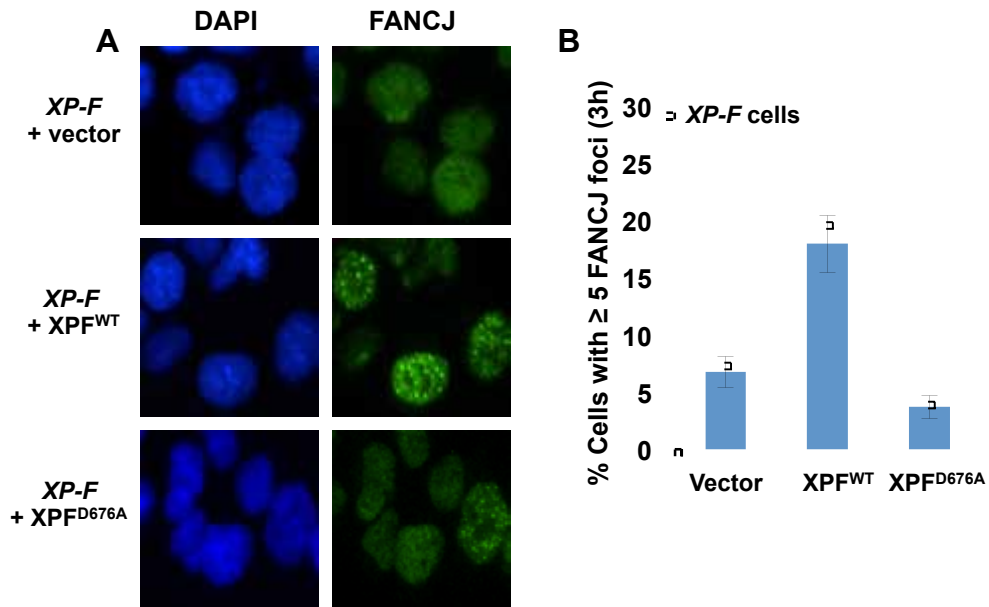
2-2 FANCD recruitment to sites of local UV-induced damage (LUDs) is dependent on XPA. (A) *XP-A* cells complemented with empty vector or *XPA*^{WT} were UV irradiated through 3 μ m filters to generate LUDs and co-immunostained with the indicated Abs. (B) Quantification of *XP-A* cells with FANCD-positive LUDs. Asterisks denote significance from student's two-tailed, unpaired t-test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$.



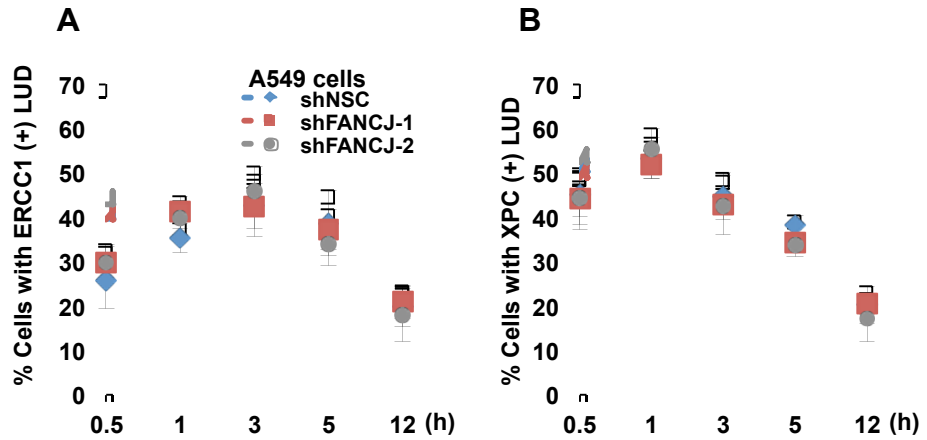
2-3 FANCI recruitment to sites of local UV-induced damage (LUDs) is dependent on XPF catalytic activity (A) *XP-F* cells complemented with empty vector, XPF^{WT}, or XPF^{D676A} were UV irradiated through 3 μ m filters to generate LUDs and co-immunostained with the indicated Abs. (B) Quantification of *XP-F* cells with FANCI-positive LUDs.



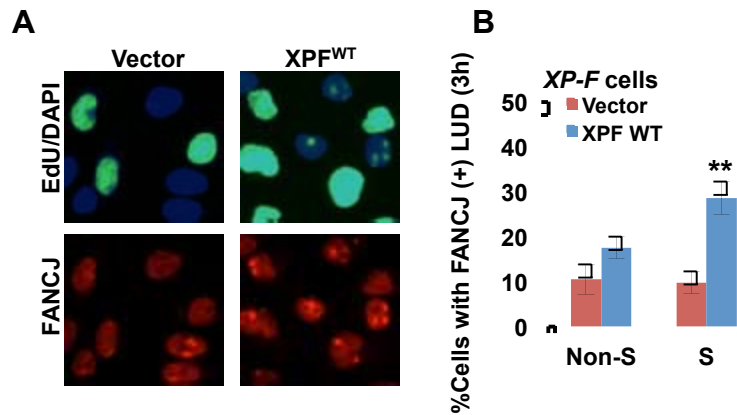
2-4 FANCI recruitment to sites of local UV-induced damage (LUDs) is dependent on XPG catalytic activity (A) *XP-G* cells complemented with empty vector *XPGE*^{WT}, or *XPGE*^{E791A} were UV irradiated through 3 μ m filters to generate LUDs and co-immunostained with the indicated Abs. (B) Quantification of *XP-G* cells with FANCI-positive LUDs.



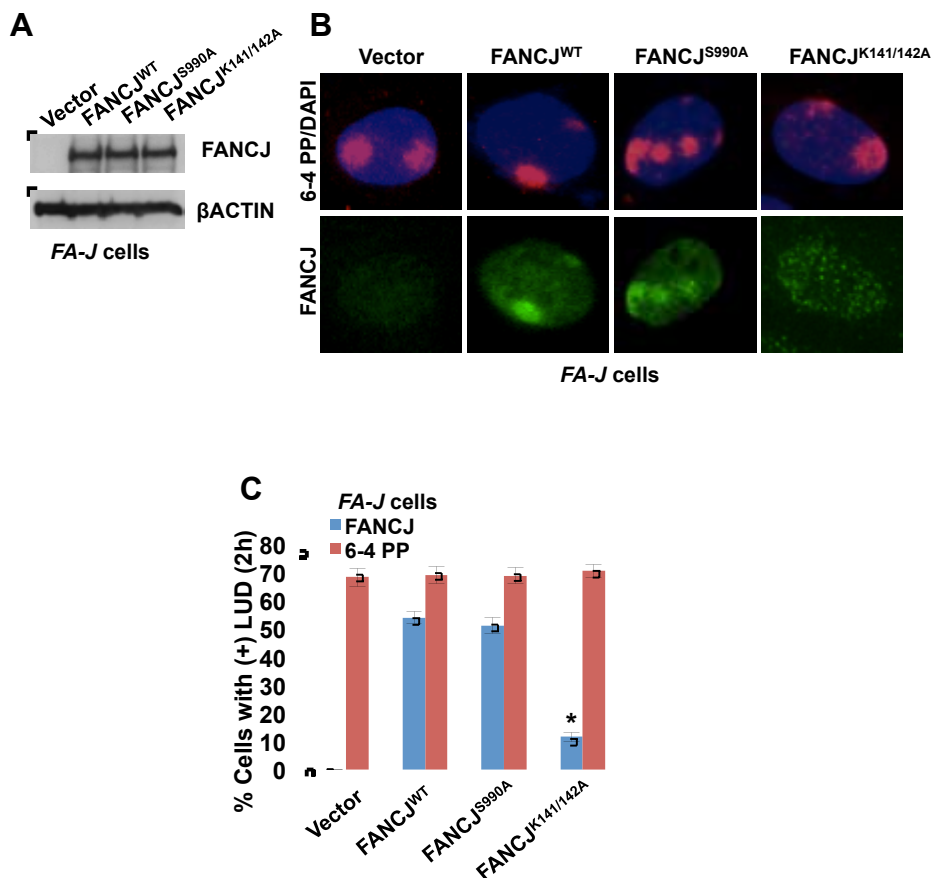
2-5 FANCI focal accumulation in response to global UV-irradiation is dependent on XPF catalytic activity (A) *XP-F* cells complemented with empty vector, *XPFWT*, or *XPFD676A* were UV irradiated and co-immunostained with the indicated Abs. (B) Quantification of *XP-F* cells with FANCI-positive foci.



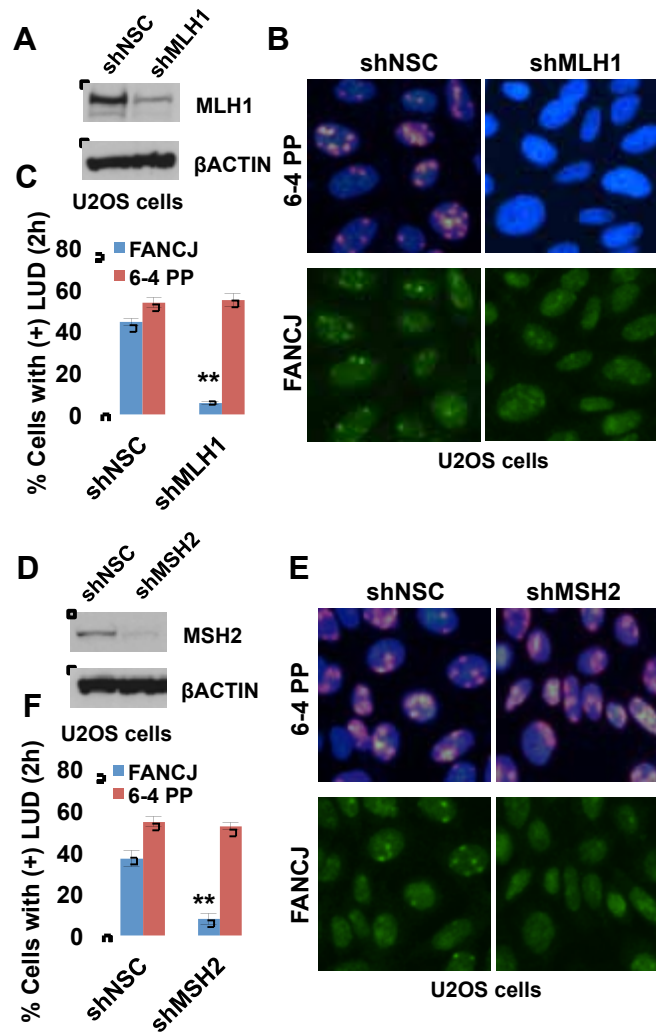
2-6 NER factors are recruited to sites of local UV-induced damage (LUDs) independently of FANCJ (A) A549 cells containing unique shRNA vectors targeting FANCJ or NSC were UV irradiated through 5 μ m filters, co-immunostained with the indicated Abs at several time points and quantified for cells with ERCC1 positive LUDs and (B) XPC positive LUDs.



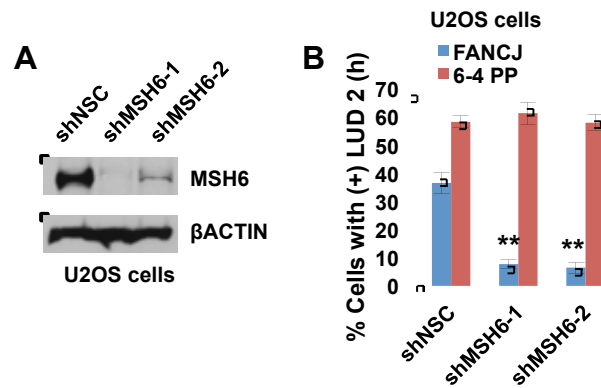
2-7 NER dependent FANCI recruitment to sites of local UV-induced damage (LUDs) occurs predominantly in S phase
 (A) *XP-F* cells complemented with empty vector or XPF^{WT} were UV irradiated through 5 μ m filters, incubated with EdU, and co-immunostained with the indicated Abs. (B) Quantification of *XP-F* cells with FANCI-positive LUDs.



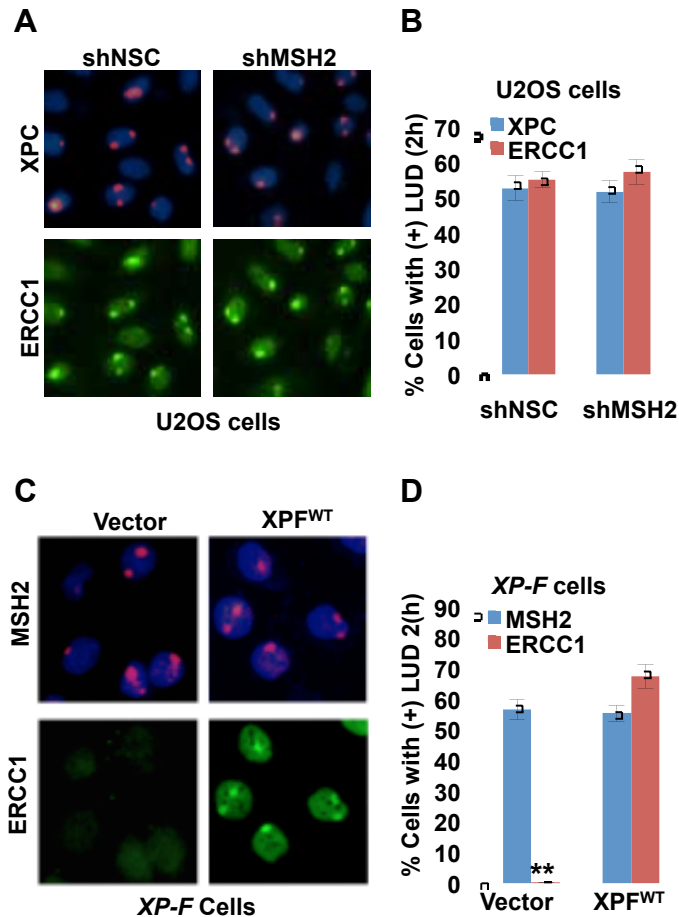
2-8 FANCJ recruitment to LUDs is dependent on MLH1 interaction and independent of BRCA1 interaction. (A) FA-J cells were complemented with empty vector, FANCJ^{WT}, FANCJ^{S990A}, or FANCJ^{K141/142A} and analyzed by immunoblot. (B) FA-J cells were UV irradiated through 5 μ m filters, co-immunostained with the indicated Abs, and quantified for FA-J cells with (C) FANCJ- and 6-4 PP-positive LUDs.



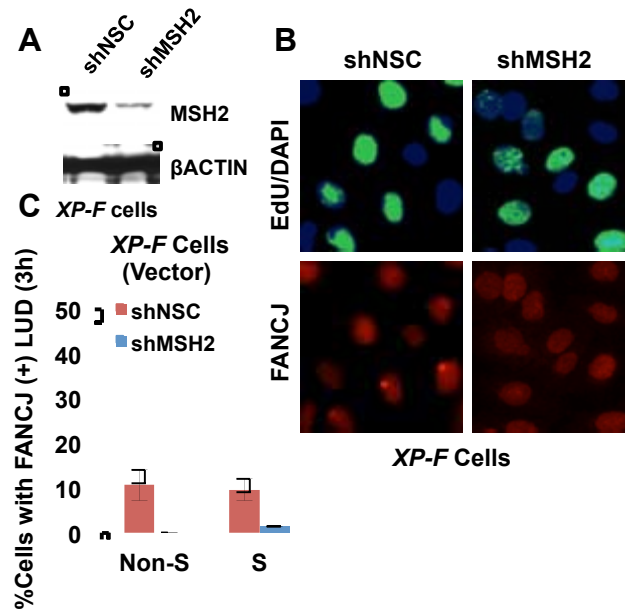
2-9 FANCDJ recruitment to LUDs is dependent on mismatch repair factors MLH1 and MSH2 (A) U2OS cells containing shRNA vectors targeting MLH1 or NSC were analyzed by immunoblot and (B) UV irradiated through micropore filters and (C) quantified for cells with FANCDJ- or 6-4 PP-positive LUDs. (D) U2OS cells containing shRNA vectors targeting MSH2 or NSC were analyzed by immunoblot and (E) UV irradiated through micropore filters and (F) quantified for cells with FANCDJ- or 6-4 PP-positive LUDs.



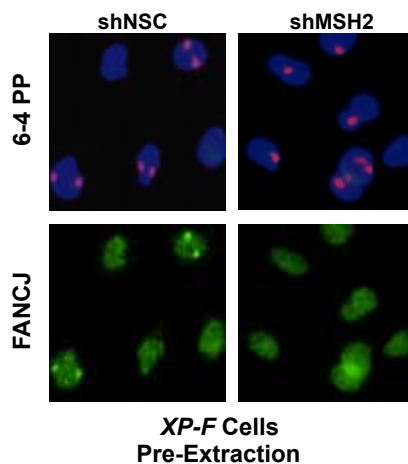
2-10 FANCI recruitment to LUDs is dependent on the mismatch repair factor MSH6 (A) U2OS cells containing shRNA vectors targeting MSH6 or NSC were analyzed by immunoblot and (B) UV irradiated through micropore filters and quantified for cells with FANCI- or 6-4 PP-positive LUDs.



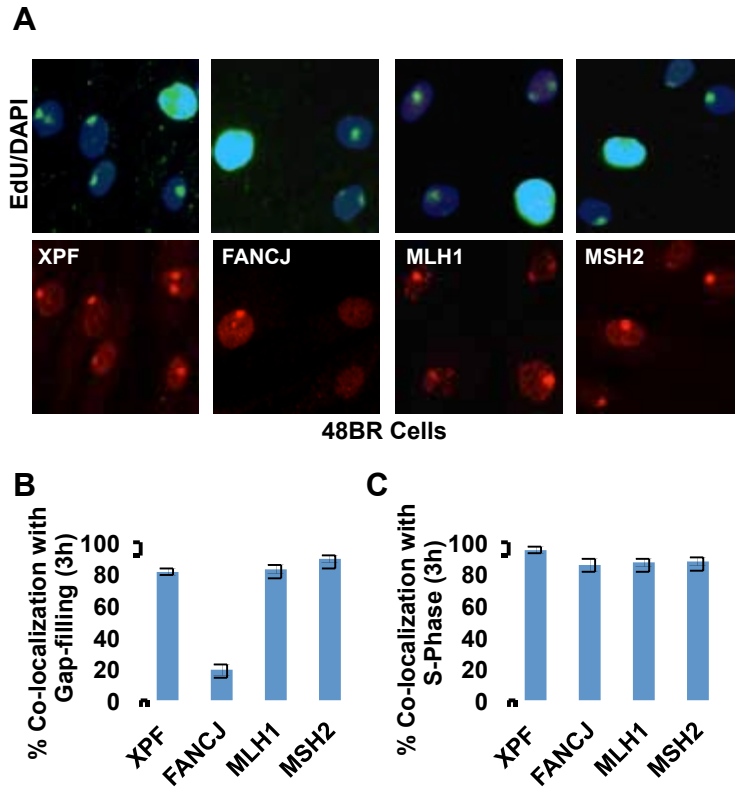
2-11 MMR and NER factors are independently recruited to sites of local UV-induced damage (LUDs) (A) U2OS cells containing shRNA vectors targeting MSH2 or NSC were UV irradiated through 5 μ m filters and co-immunostained with XPC and ERCC1 abs. Representative images are shown 2 h after UV irradiation. (B) Quantification of cells with XPC- or ERCC1-positive LUDs. (C) *XP-F* cells complemented with empty vector or XPF^{WT} were UV irradiated through 5 μ m filters and co-immunostained with MSH2 and ERCC1 abs. Representative images are shown 2 h after UV irradiation. (D) Quantification of *XP-F* cells with MSH2- or ERCC1-positive LUDs.



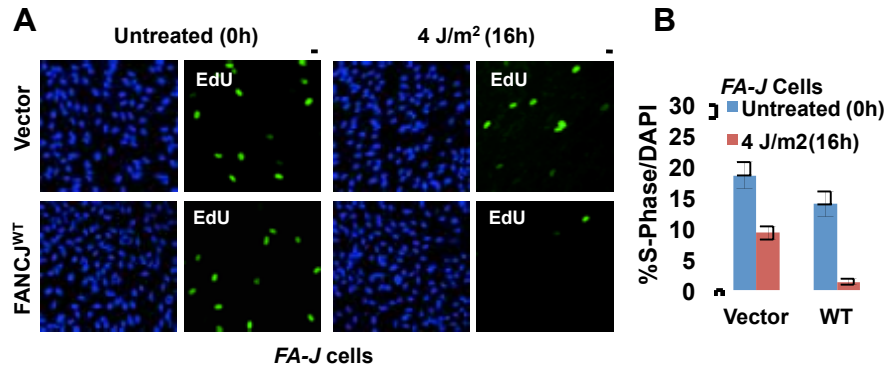
2-12 Residual FANCD1 accumulation at LUDs in *XP-F* cells during S phase is eliminated by depletion of MSH2 (A) *XP-F* cells complemented with empty vector were stably depleted of MSH2 vs. NSC and analyzed by immunoblot. (B) Cells were UV irradiated through 5 μ m filters, incubated with EdU, and co-immunostained with the indicated Abs. (C) Quantification of *XP-F* cells with FANCD1-positive LUDs.



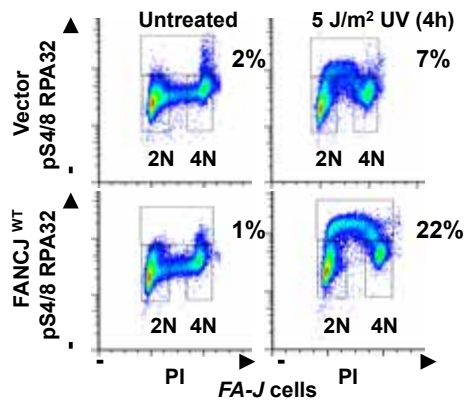
2-13 MSH2 depletion does not perturb FANCD1 chromatin localization *XP-F* cells containing shRNA vectors targeting MSH2 or NSC were UV irradiated through micropore filters, pre-extracted with 0.5% Triton-X in PBS prior to fixation, and co-immunostained with the indicated Abs, a representative image is shown 2 hrs post UV irradiation.



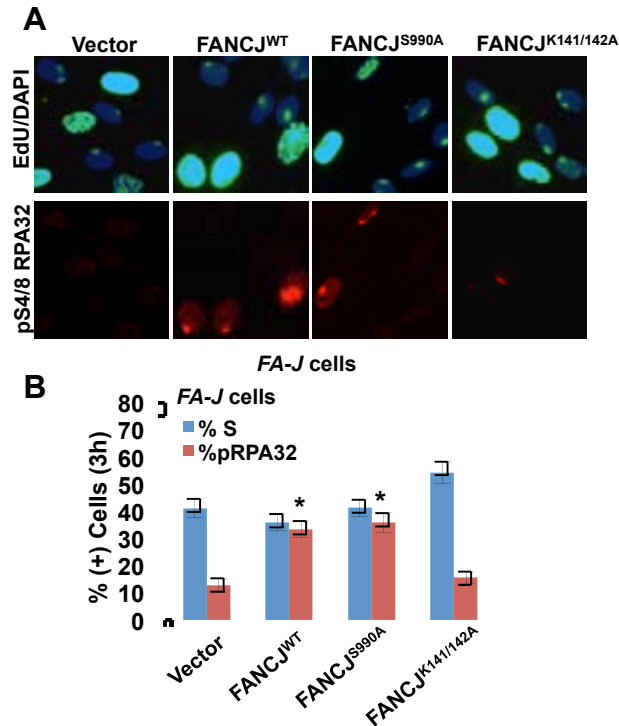
2-14 FANCI co-localizes with UV induced LUDs predominantly in S phase (A) 48BR cells were UV irradiated through 5 μ m filters prior to incubation with 10uM EdU for 3 hrs and co-immunostained with the indicated Abs. (B) Quantification of co-localization of XPF, FANCI, MLH1, or MSH2 with LUDs in non-S phase cells representing sites of gap filling and (C) quantification of co-localization of XPF, FANCI, MLH1, or MSH2 with LUDs in S-phase cells.



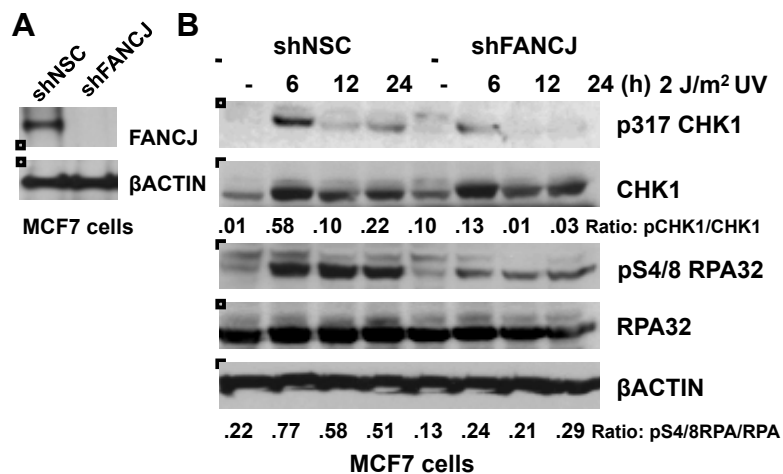
2-15 FANCI contributes to the arrest of DNA synthesis in response to UV irradiation (A) FA-J cells complemented with empty vector or FANCI^{WT} were left untreated and pulsed for 45 minutes with 10 μ M EdU or globally UV irradiated and pulsed for 45 minutes with 10 μ M EdU 16 h later. Cells were processed for EdU incorporation and co-stained with DAPI. (B) Quantification of EdU incorporation/total number of DAPI (+) cells. ≥ 1000 DAPI cells were quantified for each experiment in triplicate.



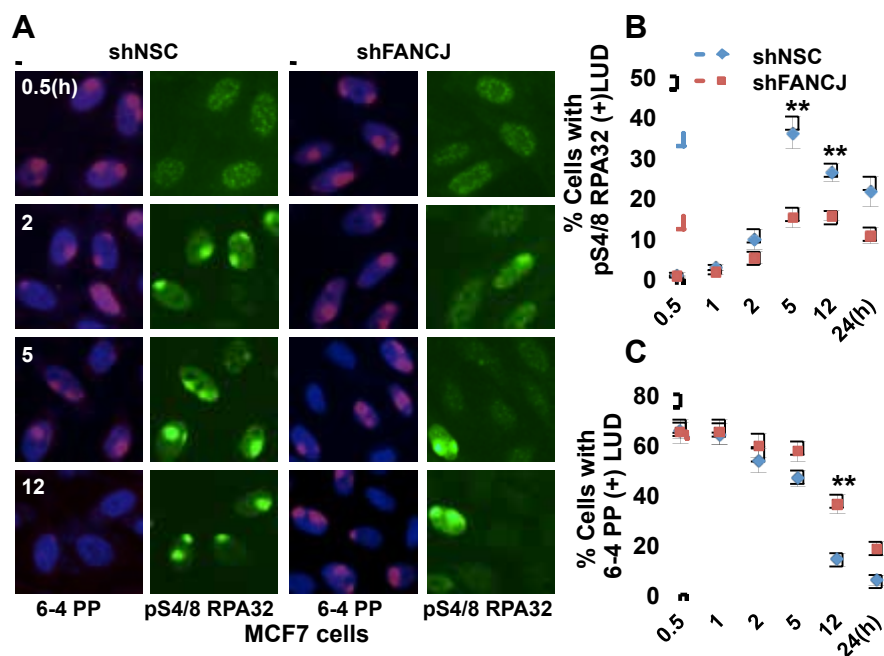
2-16 FANCI contributes to phosphorylation of RPA FA-J cells complemented with empty vector or FANCI^{WT} were left untreated or UV-irradiated and analyzed by FACS sorting for pS4/8 RPA32 positive cells, representative plots are shown.



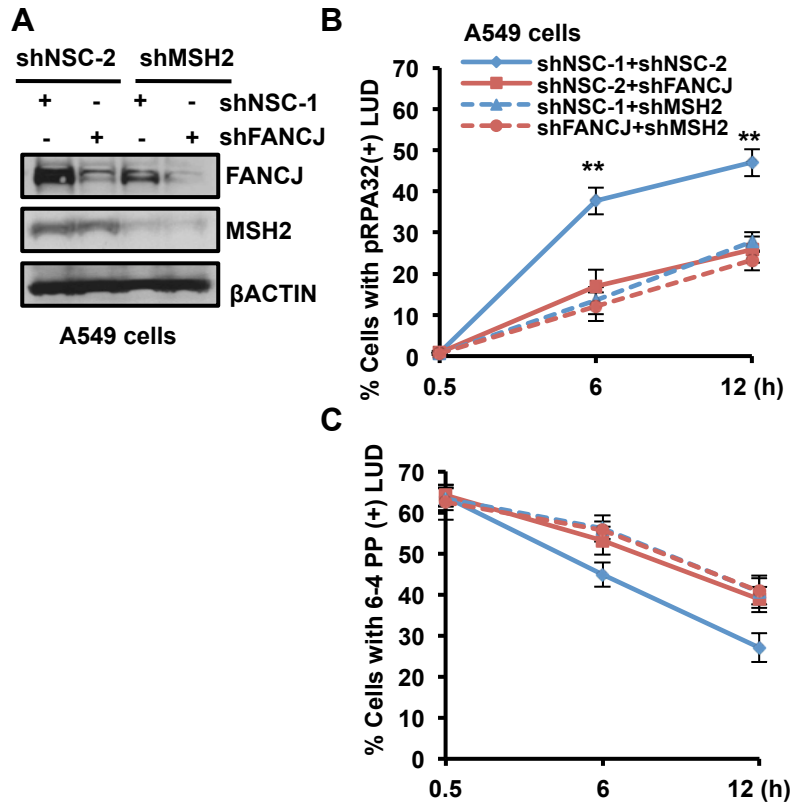
2-17 FANCI contributes to phosphorylation of RPA during S phase (A) FA-J cells complemented with empty vector or FANCI^{WT} were UV irradiated through 5 μ m filters, incubated with 10uM EdU for 3 h, and co-immunostained with phospho-S4/8 RPA32 Ab. (B) Quantification of cells with phospho-S4/8 RPA32 positive LUDs and S-phase cells.



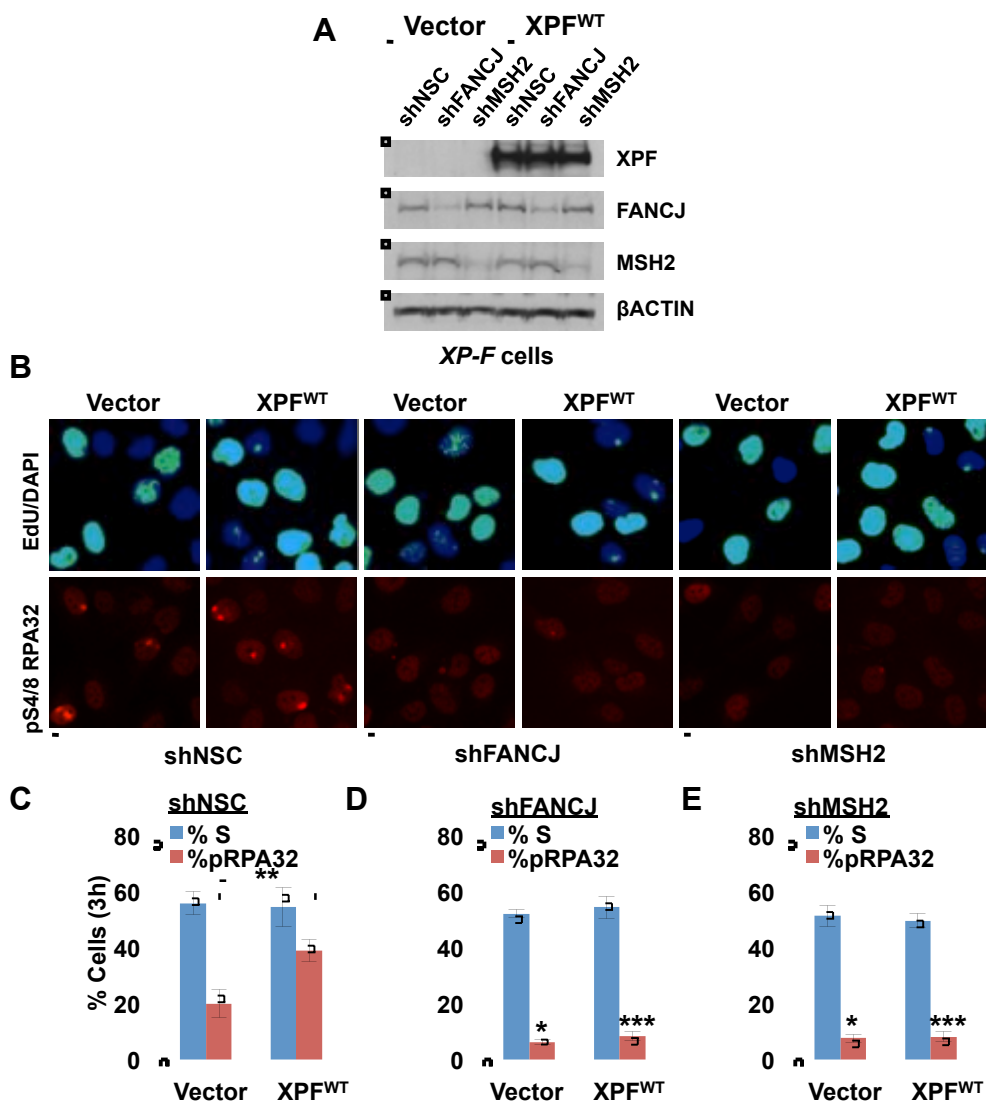
2-18 FANCJ contributes to the UV induced checkpoint. (A) Whole cell extracts of MCF7 cells containing shRNA vectors targeting FANCJ or NSC were analyzed by immunoblot and (B) were analyzed by immunoblot with the indicated Abs at the indicated time points after UV irradiation. The ratio of phospho-protein/total protein by densitometry using Image J software is quantified.



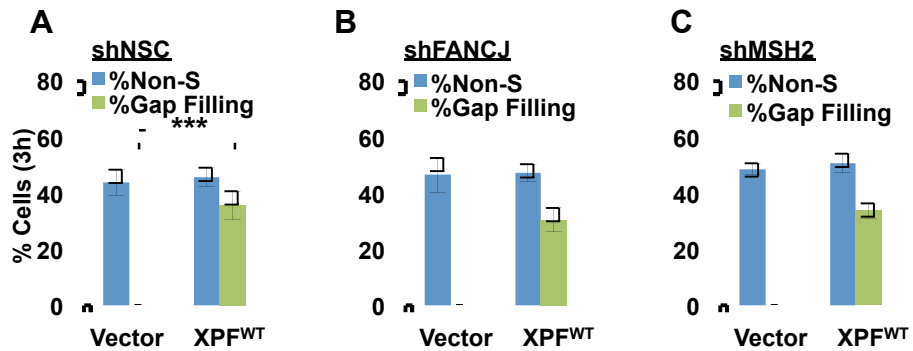
2-19 FANCJ contributes to the UV induced checkpoint and elimination of UV induced lesions. (A) MCF7 cells containing shRNA vectors targeting FANCJ or NSC were UV irradiated through 5 μ m filters to generate LUDs and co-immunostained with the indicated Abs at several time points. (B) Quantification of cells with phospho-S4/8 RPA32-positive LUDs. (C) Quantification of 6-4 PP-positive LUDs.



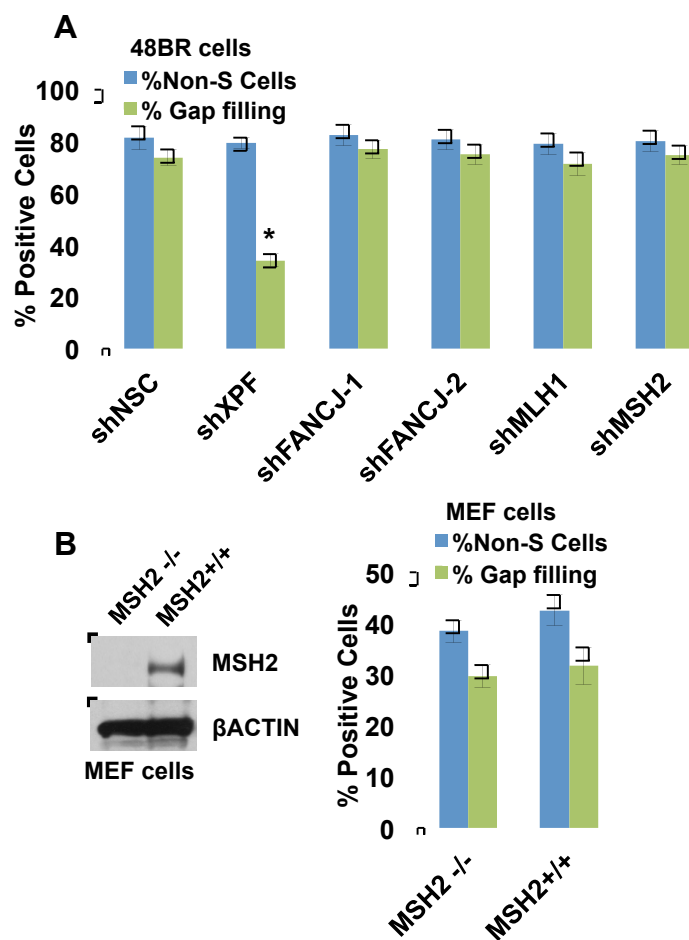
2-20 FANCJ and MMR function in a common pathway for RPA phosphorylation and 6-4 Photoproduct elimination (A) A549 cells containing shRNA vectors targeting FANCJ or NSC were stably depleted of MSH2 versus a second NSC and analyzed for immunoblot or (B) UV irradiated through 5 μ m filters to generate LUDs and co-immunostained with the indicated Abs at several time points and quantified for cells with phosphoserine4/8 RPA32-positive LUDs and (C) 6-4 PP-positive LUDs.



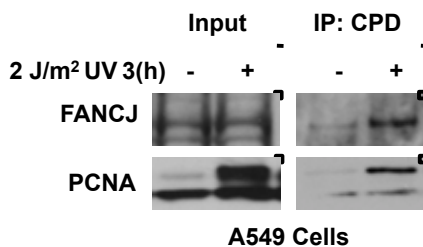
2-21 FANCI and MSH2 are required for RPA phosphorylation in S phase (A) *XP-F* cells complemented with empty vector or *XPF*^{WT} were stably depleted of FANCI, MSH2, or NSC using shRNA vectors and analyzed by immunoblot. (B) Cells were UV irradiated through 5 μ m filters, incubated with EdU, and co-immunostained with the indicated Abs. (C) Quantification of phospho-S4/8 RPA32 positive LUDs in S phase cells expressing shNSC (D) shFANCI and (E) shMSH2.



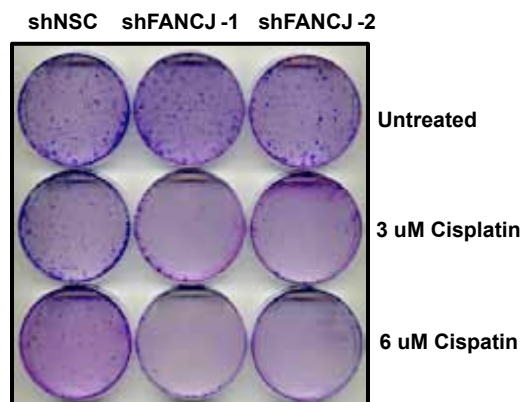
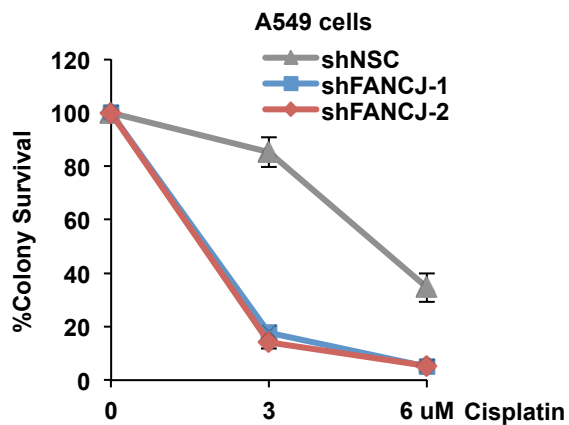
2-22 FANCI and MSH2 are not required for NER dependent gap filling (A) XP-F cells complemented with empty vector or XPF^{WT} were stably depleted of FANCI, MSH2, or NSC using shRNA vectors and cells were UV irradiated through 5 μ m filters, incubated with EdU, and co-immunostained. Quantification of NER dependent gap filling in non-S phase cells expressing shNSC (B) shFANCI and (C) shMSH2.



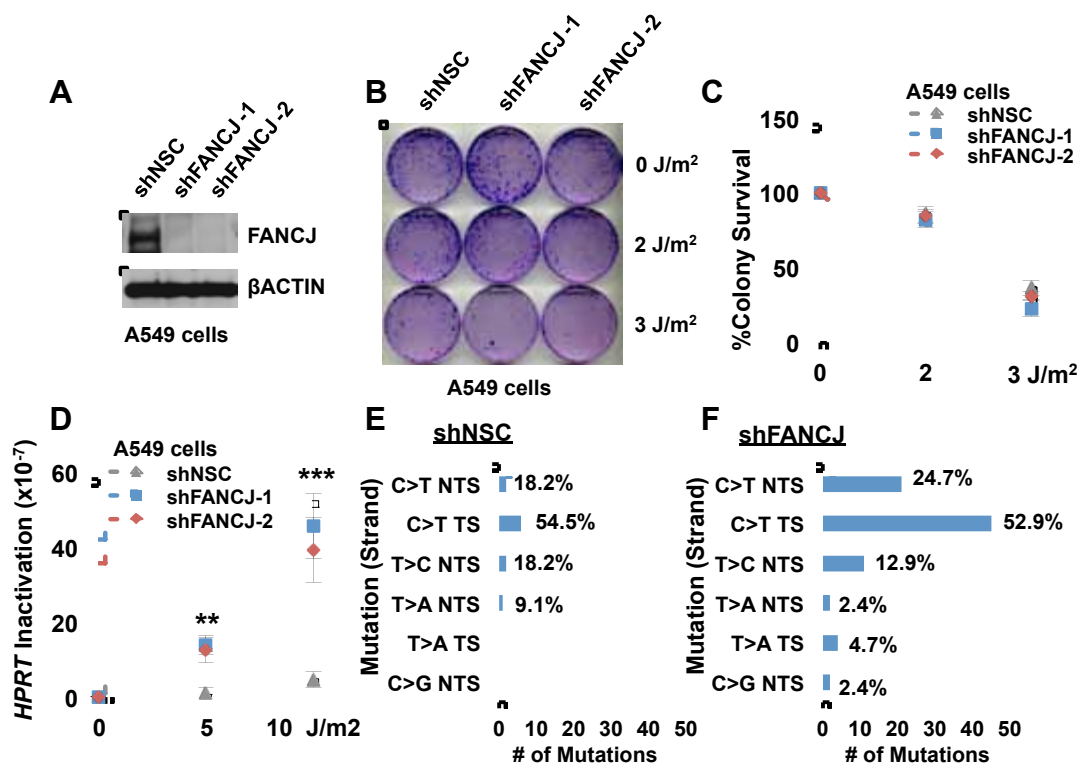
2-23 FANCJ and MMR factors are not required for NER dependent gap filling (A) 48BR cells containing shRNA vectors targeting XPF, FANCJ-1, FANCJ-2, MLH1, MSH2 or NSC were UV irradiated through micropore filters prior to incubation with 10uM EdU for 3 hrs and quantified for gap-filling in non-S cells. (B) MSH2 isogenic mouse embryonic fibroblasts were analyzed by immunoblot, treated as in A, and quantified for gap filling in non- S phase cells.



2-24 FANCJ precipitates with UV modified PCNA and CPD from chromatin extracts. A549 cells were left untreated or globally UV irradiated and collected in 150 nM NETN buffer and sonicated. The sonicated fraction was spun down and the lysate was used for input or for CPD immuno-precipitation (IP). IPs were then analyzed by immunoblot with the indicated Abs.

A**B**

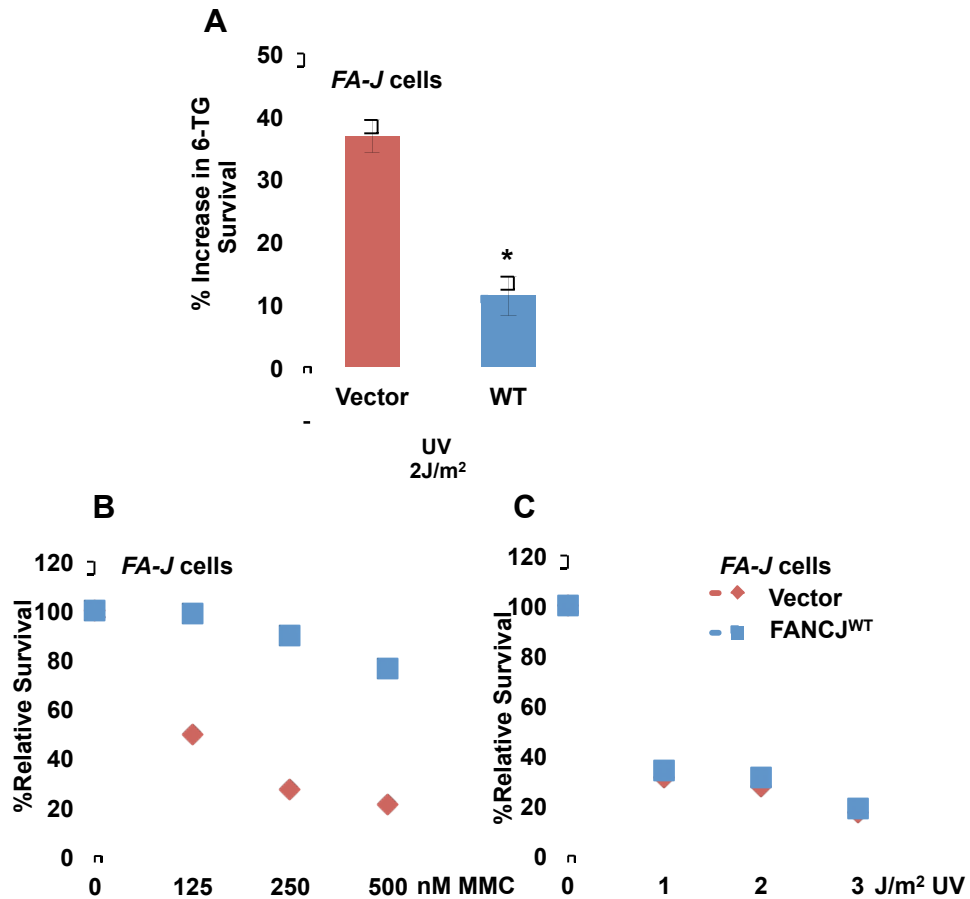
2-25 FANCJ deficient cells are sensitive to the DNA interstrand crosslinking agent cisplatin (A) A549 cells expressing shRNA vectors targeting FANCJ or NSC were left untreated or treated with Cisplatin and analyzed for colony survival. (B) Quantification of surviving colonies.



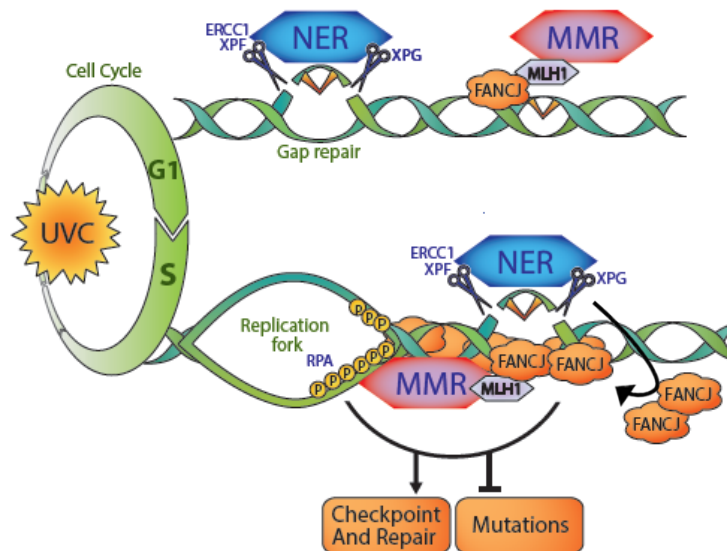
2-26 FANCI suppresses UV-induced mutations. (A) A549 cells expressing individual shRNA vectors targeting FANCI or NSC were analyzed by immunoblot and (B) left untreated or globally UV irradiated and analyzed for colony survival. (C) Quantification of surviving colonies. (D) Quantification of 6-thioguanine (6-TG)-resistant HPRT mutant colonies from mutagenesis assay. (E) Quantification of the distribution of HPRT-inactivating mutations in A549 cells expressing shRNA to NSC and (F) or shRNAs to FANCI (combined).

#Clones	Position (Strand)	(Sequence Change (5'→3'))	Exon	Amino Acid
shNSC				
2	97(-)	CTTT(C>T)CAAA	2	Glu->Lys
1	112(+)	TATT(C>T)CTCA	2	Pro->Ser
1	260(-)	ATTT(C>T)TATT	3	Arg->Lys
3	400(-)	TCTT(C>T)CACA	5	Glu->Lys
1	443(+)	CTTT(C>T)CTTG	6	Ser->Phe
1	374(+)	ACTT(T>C)AACT	4	Leu->Ser
1	297(+)	ATTT(T>A)ATCA	3	Phe->Leu
1	374(+)	ACTT(T>A)AACT	4	Leu->Stop
shFANCJ 1+2				
5	112(+)	TATT(C>T)CTCA	2	Pro->Ser
4	145(+)	ACGT(C>T)TTGC	3	Leu->Phe
1	149(+)	CTTG(C>T)TCGA	3	Ala->Val
3	212(-)	ATAG(C>T)CCCC	3	Gly->Asp
1	227(+)	TTTG(C>T)TGAC	3	Ala->Val
9	260(-)	ATTT(C>T)TATT	3	Arg->Lys
7	272(-)	GGAT(C>T)TATC	3	Arg->Lys
3	275(+)	AGAT(C>T)CATT	3	Ser->Phe
2	280(+)	CATT(C>T)CTAT	3	Pro->Ser
1	281(+)	ATTC(C>T)TATG	3	Pro->Leu
3	364(+)	TGAT(C>T)TCTC	4	Leu->Phe
1	379(-)	TTTC(C>T)AGTT	4	Gly->Arg
2	380(-)	CTTT(C>T)CAGT	4	Gly->Glu
6	400(-)	TCTT(C>T)CACA	5	Glu->Lys
2	412(-)	GTGT(C>T)AATT	6	Asp->Asn
1	443(+)	CTTT(C>T)CTTG	6	Ser->Phe
7	500(-)	GGTC(C>T)TTTT	7	Arg->Lys
3	509(-)	ACTT(C>T)GTGG	7	Arg->Gln
5	544(-)	ATTT(C>T)AAAT	8	Glu->Lys
3	224(+)	TTCT(T>C)TGCT	3	Phe->Ser
2	296(+)	GATT(T>C)TATC	3	Phe->Ser
2	367(+)	TCTC(T>C)CAAC	4	Ser->Pro
3	437(+)	ACTT(T>C)GCTT	6	Leu->Ser
1	532(+)	AGAC(T>C)TTGT	7	Phe->Leu
3	263(-)	ACTA(T>A)TTTC	3	Asn->Ile
1	407(+)	GATA(T>A)AATT	6	Ile->Lys
1	409(-)	TCAA(T>A)TATA	6	Ile->Phe
1	543(+)	GATT(T>A)GAAA	8	Phe->Leu
1	280(+)	CATT(C>G)CTAT	3	Pro->Ala
1	443(+)	CTTT(C>G)CTTG	6	Ser->Cys

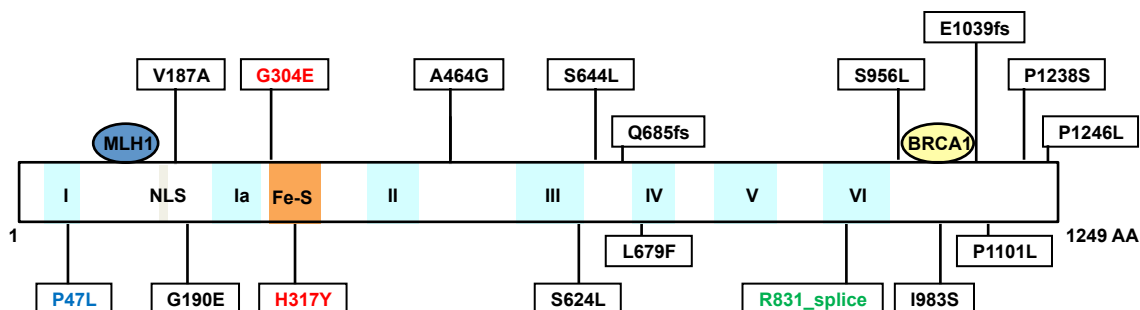
Table 2-1 List of UV-induced mutations at *HPRT* locus



2-27 FANCI suppresses UV-induced mutations (A) FA-J cells expressing empty vector control or FANCI^{WT} were UV irradiated and analyzed for survival in 6-TG relative to untreated cells (B) Empty vector control- or FANCI^{WT}-complemented FA-J cells were analyzed for relative survival after mitomycin C treatment. Cells were stained with crystal violet, solubilized, and absorbance was measured at 590nm. The relative absorbance of treated/untreated was quantified as relative survival. (C) FA-J cells were treated as in D, except analyzed for relative survival after UV irradiation.



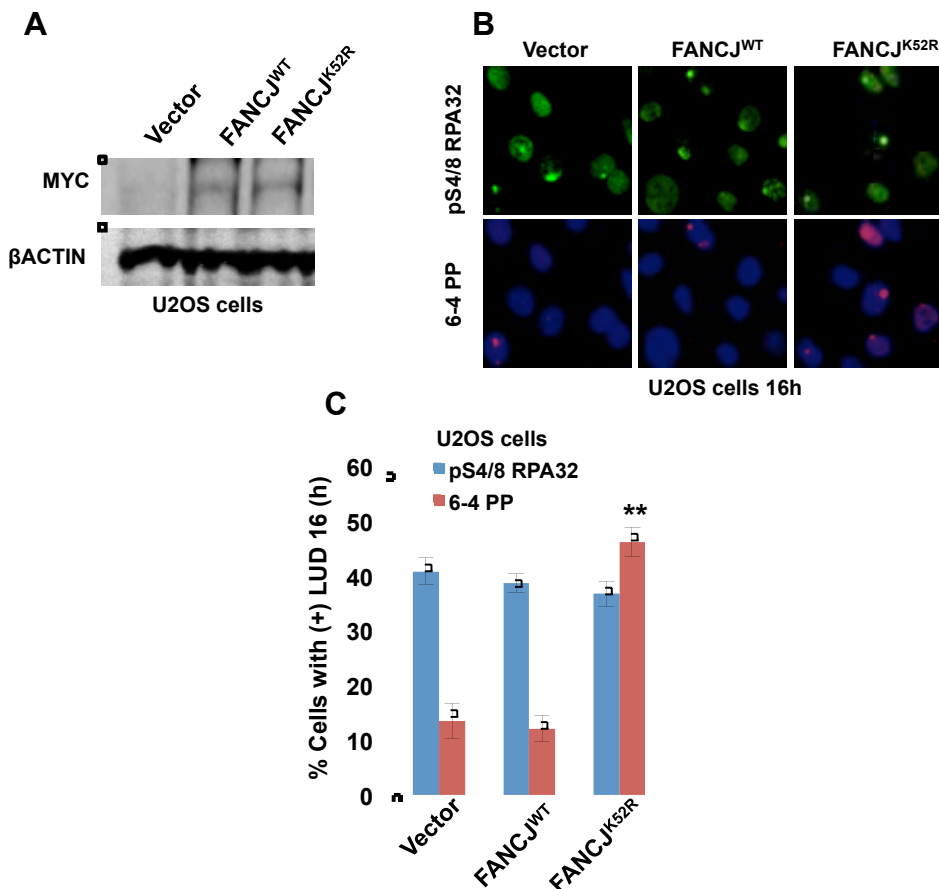
2-28 FANCD1 functions in response to UV irradiation Model of FANCD1 function in response to UV-irradiation. NER and MMR factors are recruited to sites of local UV induced damage in non-S phase cells where NER, but not MMR, is required for gap filling. In S-phase cells, both NER and MMR factors contribute to the accumulation of FANCD1. MMR through MLH1 binding localizes FANCD1 to sites of UV induced damage. NER incision enhances the accumulation of FANCD1 at the lesion site. Collectively, these events ensure a robust checkpoint response to limit the replication of damaged DNA, induction of mutations, and cancer.



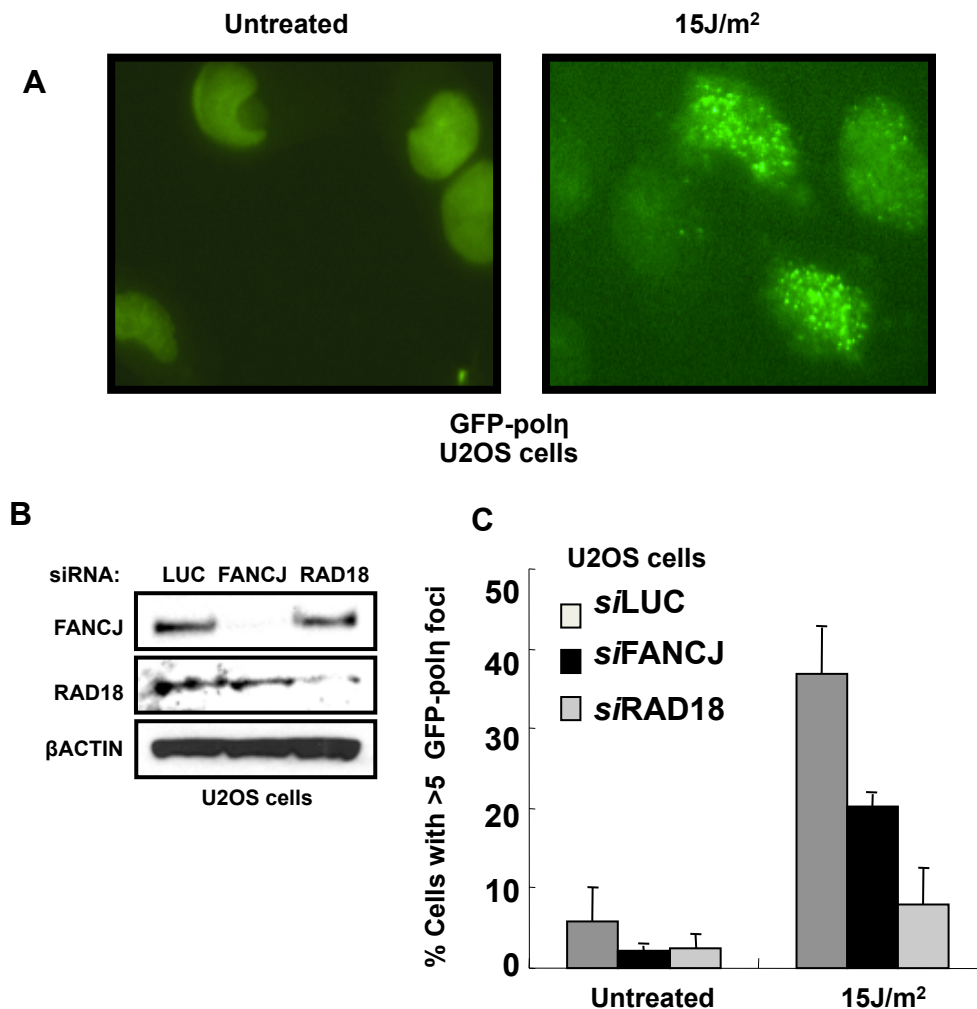
2-29 Mutations in FANCD1 and MMR loci occur in melanoma genomes FANCD1 protein coding mutations were identified from sequenced melanoma genomes in cBioPortal and the Catalogue of Somatic Mutations in Cancer (CoSMiC) databases. Mutations are located throughout the FANCD1 sequence as indicated. Two specific amino acids were previously identified, in hereditary breast cancer (blue) and in Fanconi Anemia (green). Mutations found in the iron-sulfur domain (red). Sites where FANCD1 interacts with MLH1 (K141/K142) and BRCA1 (S990) are indicated.

FANCI		MSH2	
P47L	Missense	Q395*	Nonsense
V187A	Missense	P476S	Missense
G190E	Missense	L478F	Missense (Squamous)
G304E	Missense	R680*	Nonsense
H317Y	Missense	C822F	Missense
A464G	Missense	MSH6	
S624L	Missense	K155R	Missense
S644L	Missense	R240*	Nonsense
L679F	Missense	G306K	Missense
Q685*	Nonsense	A457V	Missense
R831_splice	Splice Site	P623L	Missense
I983S	Missense	S677I	Missense
S956L	Missense	T757I	Missense
E1039fs	Frameshift	M868I	Missense
P1101L	Missense	Q939*	Nonsense
P1238S	Missense	T1008I	Missense
P1246L	Missense	T1219I	Missense
P47L	Missense	PMS2	
V187A	Missense	S769F	Missense
G190E	Missense	K178R	Missense
MLH1		S769F	Missense
R217C	Missense	R151C	Missense
H315Y	Missense	S128L	Missense
T347I	Missense	H634Y	Missense
S368L	Missense	H552R	Missense
L507*	Nonsense		
L521fs	Frameshift		
p.? (c.1732-1G>A)	Intronic		

Table 2-2 List of Mutations in FANCI and MMR loci from melanoma genomes protein coding mutations were identified from sequenced melanoma genomes in cBioPortal and the Catalogue of Somatic Mutations in Cancer (CoSMiC) databases.



2-30 Ectopic expression of catalytic inactive FANCI^{K52R} disrupts clearance of UV induced lesions in U2OS cells (A) U2OS cells were transfected with pCDNA3 constructs containing vector, FANCIWT, or FANCIK52R and analyzed by immunoblot with the indicated Abs. (B) Cells were UV irradiated through 5 μm filters to generate LUDs and co-immunostained with the indicated Abs 16h post treatment and (C) quantified for cells with phospho-serine4/8 RPA32-positive LUDs and 6-4 PP-positive LUDs.



2-31. FANCJ expression promotes UV induced GFP-polη foci formation in U2OS cells (A) U2OS cells were transfected with GFP-polη and left untreated or globally UV irradiated and analyzed on a fluorescent microscope. (B) Cells were co-transfected with GFP-polη and siRNA reagents targeting luciferase control, FANCJ, or RAD18 and analyzed by immunoblot with the indicated Abs. (C) Cells were left untreated or globally UV irradiated quantified for cells with >5 GFP-polη foci.

Conclusions

Here, we show that both NER and MMR proteins promote the localization of the FANCD1 DNA helicase to sites of UV-induced lesions to ensure a robust S phase checkpoint response. MMR proteins initially recruit FANCD1 and its further accumulation requires dual incision by the NER endonucleases XPD and XPG (Figures 2-2, 2-3, 2-4). Although FANCD1-deficiency does not cause UV-induced sensitivity, this analysis revealed an important role for FANCD1 in promoting an S phase checkpoint response, lesion repair, and suppressing UV-induced mutations (Figures 2-15, 2-16, 2-17, 2-18, 2-19). Consistent with FANCD1 and MMR functioning in a common pathway, we found that FANCD1 or MMR deficiency alone or in combination generated similar defects (Figure 2-20). Correspondingly, the direct interaction between MLH1 and FANCD1 is essential for both FANCD1 localization and function at sites of UV-induced damage, whereas the BRCA1 interaction is not required (Figure 2-8). Similar to NER, MMR proteins localize to LUDs in both non-S and S phase cells whereas FANCD1 is predominantly found at sites of UV-damage in S phase cells (Figure 2-14). Together, this work demonstrates that distinct pathways merge in S phase cells to ensure a robust UV-induced DNA damage response.

These findings are important in light of the fact that defects in MMR have been associated with skin cancers found in the HNPCC variant Muir-Torre syndrome (Kruse et al. 1998; Suspiro et al. 1998; Mathiak et al. 2002).

Furthermore, we searched for both FANCD1 and MMR mutations within sequenced melanoma genomes using cBioPortal and the Catalogue of Somatic Mutations in Cancer (CoSmiC) database (Cerami et al. 2012; Gao et al. 2013; Forbes et al. 2011). We identified mutations in FANCD1, MSH2, MSH6, MLH1, and PMS2 (Figure 2-29, Table 2-2). The majority of FANCD1 mutations target the helicase domain including domains important for enzyme function, such as the Fe-S domain and helicase boxes III, IV, and V (Figure 2-29). In addition, some of the mutations have been detected previously. The FANCD1^{P47} residue was targeted in breast cancer and was shown to be ATPase and helicase inactive *in vitro* (Cantor et al. 2004). The splice mutant FANCD1^{R831} is an allele in FA and eliminates conserved helicase boxes required for enzyme function (Cantor et al. 2004; Gupta et al. 2005; Cantor & Guillemette 2011). To determine if loss of FANCD1 ATPase/helicase/translocase activity disrupts the UV response, we attempted to express the catalytic inactive FANCD1^{K52R} mutant in FA-J cells. Because FANCD1 deficient FA-J cells are defective in the UV response and the FANCD1^{K52R} mutant has weak expression compared to FANCD1^{WT}, it was unclear if the mutant was defective in complementing FA-J cells or mediating the UV response. Thus, we overexpressed the FANCD1^{K52R} mutant in U2OS cells. Here, we found significant defects in lesion clearance at 16 h following UV damage, but no significant affect on RPA phosphorylation at this time point (Figure 2-30). Thus, loss of FANCD1 expression could disrupt checkpoint activation, whereas expression of an enzyme inactive mutant could dominantly disrupt repair.

Further supporting that multiple pathways contribute to high fidelity repair after UV irradiation, similar to skin tumors from XP patients, MMR deficient and FANCD1-deficient cells display an elevated frequency of UV-induced C>T point mutations (Table 2-1) (Dumaz et al. 1993; Borgdorff et al. 2006). Similar to FANCD1 deficiency, MMR deficiency also has modest effects on UV sensitivity despite reduced checkpoint and apoptotic responses (Yoshino et al. 2002; van Oosten et al. 2005). Thus, we propose that FANCD1 intersects MMR and NER dependent repair pathways to promote efficient checkpoint activation, lesion clearance, and suppress UV-induced mutations (Figure 2-28). Conceivably, in the absence of FANCD1 and its checkpoint function error-prone polymerases induce mutations at sites of UV induced lesions. Indeed, the high-fidelity TLS polymerase, pol η has reduced foci formation in response to global UV irradiation in FANCD1 deficient cells (Figure 2-31).

The NER factor XPA contributes to the S phase checkpoint following UV-induced irradiation. However, not all NER factors are required suggesting that this checkpoint function is distinct from NER repair in G1 phase (Gillet & Schärer 2006). These findings further suggest that XPD promotes RPA phosphorylation in S phase cells (Figure 2-21). Interestingly, XPD is the FA gene, FANCD1 (Bogliolo et al. 2013; Kashiwaga et al. 2013). Given that XPD promotes FANCD1 accumulation in S phase cells, this data also suggests that FANCD1 functions downstream of this FA factor to promote RPA phosphorylation throughout S

phase. NER dependent incision may provide a better substrate or change the DNA structure, enabling distribution of FANCDJ at the lesion site (Figure 2-28). Here, FANCDJ could facilitate repair of lesions ahead of the replication fork through checkpoint induction and the arrest of DNA synthesis to limit mutation induction. Conceivably this function is shared by FANCDJ partners, such as Bloom's syndrome helicase (BLM), or the FA pathway, explaining its link to the UV response and checkpoints that limit genomic instability (Suhasini et al. 2011; Kelsall et al. 2012; Singh et al. 2009; Nalepa et al. 2013). It has long been proposed that ATR-BLM and FA pathway interactions maintain the genome by restoring productive replication following replication stress (Davies et al. 2004; Olson et al. 2006; Sobeck et al. 2006).

The two-step mobilization of FANCDJ to UV-induced lesions, localization by MMR and further accumulation after NER dependent post-incision could ensure pathway coordination. Indeed, the combined loss of NER and MMR enhances UV-induced mutagenesis (Nara et al. 2001). While MMR and NER proteins have been shown to have overlapping substrates, it remains to be determined whether they bind the same or a distinct type of UV lesion (Zhao et al. 2009). FANCDJ loading by MLH1 is reminiscent of the requirement of the bacterial MutL, homologous to the MutL α complex, for loading helicase II (UvrD) onto DNA (Mechanic et al. 2000). Helicase II functions with DNA polymerase I to release oligonucleotide fragments containing UV photoproducts (Caron et al. 1985). In

contrast to Helicase II, our data do not support a role for FANCD1 or MMR in gap repair. However, these findings do not exclude the possibility that MMR and FANCD1 contribute to the fidelity of NER-dependent gap filling. Alternatively, loading of FANCD1 by MMR factors could unwind and disrupt secondary DNA structures that impede NER processing. Indeed, MMR factors bind secondary structures such as G-4 quadruplex DNA that FANCD1 unwinds (Larson et al. 2005; Wu et al. 2008). Our group also previously reported that FANCD1 depends on MLH1 for localization to sites of DNA interstrand crosslinks (Suhasini et al. 2013).

Collectively, the data presented in this manuscript provide a framework for understanding the contributions of distinct DNA repair pathways to the DNA damage response to UV irradiation in human cells. The identification of a novel function for MMR in localizing FANCD1 to sites of UV induced damage could be useful for several reasons. First, a functional assay examining FANCD1 or MMR localization to LUDs could help in the discrimination between missense and pathogenic MMR variants, especially those identified by melanoma genome sequencing studies. Loss of FANCD1 localization and function could be also uniquely disrupted by MMR gene mutations as found in tumors in which canonical MMR is intact. Second, the MMR-FANCD1 pathway could represent a unique tumor suppression pathway that provides opportunities for selective therapy in effected tumors. In melanoma, loss of FANCD1 function or expression could be a consequence of not only FANCD1 mutations (Table 2-2), but also MMR

mutations. Indeed, ~5.7 % of tumors are affected by FANCI mutations, which did not co-segregate with MMR gene mutations (Cerami et al. 2012; Gao et al. 2013). Associated skin tumors may be selectively sensitive to ICL-inducing agents, which is a hallmark of FA-J patient cells. In light of the recent finding that XPD is the FA gene, FANCD1, it will be important to determine if the FA pathway has a more fundamental role in the response to UV irradiation and/or in reducing the emergence of disease (Bogliolo et al. 2013; Kashiwaga et al. 2013).

Materials and Methods

Cell Culture

A549, MCF7, and U2OS cells were cultured in DMEM (Gibco, Life Technologies) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. *FA-J*, 48BR, MEFs, GM04429 *XP-A*, XP2YO *XP-F*, and XPCS1RO *XP-G* cells and their respective complements were cultured in DMEM supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. Patient cell lines XP2YO *XP-F*, XPCS1RO *XP-G* and their respective complements were generated by Dr. Orlando Schärer and MSH2^{-/-} or ^{+/+} MEFs were a generous gift of Janet Stavnezer.

DNA constructs

FA-J cells were infected with pOZ retroviral vectors³² expressing FANCI^{WT}, FANCI^{K141/142A}, FANCI^{S990A}, or empty vector as described earlier^{23, 24}. Stable cell lines were generated by sorting with anti-IL-2 magnetic beads (Dyna Beads). The pCDNA-3myc-6xhis vectors were generated with a QuickChange site-directed mutagenesis kit (Stratagene) using published primers for FANCI^{K52R}²³. GFP-pol η was expressed in U2OS cells as described in (Kannouche et al., 2001, Watanabe et al., 2004, Xie et al., 2010).

RNA Interference

The packaging cell line 293TL was used to produce lentiviral particles containing pGIPZ or pLKO.1 vectors and 293TD cells were used to produce retroviral particles containing pStuffer vector. Cells were transfected with 1:1:2 ug of DNA packaging versus insert using Effectene[®] transfection reagent (Qiagen) 48 h prior to harvesting retroviral or lentiviral supernatants. Supernatants were filtered and added to recipient cell lines with 1 ug/ml polybrene. Cells infected with shRNA vectors were selected with either puromycin (pGIPZ, pLKO.1) or hygromycin (pStuffer). For shRNA-mediated silencing the mature antisense was used for pLKO.1

shNSC	5'CCGCAGGTATGCACGCGT3',	shMLH1
5'AATACAGAGAAAGAAGAACAC3',		shMSH2
5'AAACTGAGAGAGATTGCCAGG3',		
shXPF	5'AAATCACTGATACTCTTGCGC3',	shFANCI
5'TATGGATGCCTGTTTCTTAGCT3',	for pGIPZ	shNSC
5'ATCTCGCTTGGGCGAGAGTAAG3',		shMSH2-1
5'ATTACTTCAGCTTTTAGCT3',		shMSH2-2
5'GCATGTAATAGAGTGTGCTAA3',		shMSH6-1
TTCAACTCGTATTCTTCTGGC,	and	shMSH6-2

TTTCAACTCGTATTCTTCTGG. The pStuffer vectors were a generous gift of Dr Junjie Chen. The pStuffer shRNA targeting luciferase was 5'GUGCGCUGCUGGUGCCAAC3', shFANCI-1 5'GUACAGUACCCCACCUUAU

3', and shFANCI-2 5'GAUUUCCAGAUCCACAAUU3'. RNAi mediated depletion of Luciferase, FANCI, or RAD18 using siRNA reagents was performed as described previously (Xie et al., 2010)

Local UV irradiation and Immunofluorescence

Local UV irradiation was performed as described ³³ using a 254 nm UV lamp (UVP inc., Upland, CA) with a dose of 100 J/m² through 3 or 5 μ m Isopore polycarbonate membrane filters (Millipore). Cells were fixed for 10 min with either ice cold methanol or 3% paraformaldehyde/2% sucrose in phosphate buffered saline (PBS), permeabilized for 5 min with 0.5% Triton X-100, and treated with 0.08M NaOH for 2 min. only prior using 6-4 PP or CPD Abs. Coverslips were rinsed 3x in 1xPBS prior to each step. For immunostaining using combined cocktails of primary or secondary antibody staining, cells were incubated for 40 min. each in a humid chamber, face down on a 100 μ L meniscus of antibody cocktails diluted in 3%BSA in PBS. Primary Abs used were anti-FANCI (1:500 Sigma, Lot #051M4759, #014K4843), anti-pS4/S8 RPA32 (1:500 Bethyl), anti-MLH1 (1:200 BD Bioscience), anti-MSH2 (1:200 Calbiochem), anti-XPF (1:200 Neomarkers), anti-ERCC1 (1:500 Santa Cruz), anti-XPC (1:500 Abcam), anti-6-4 PP, and anti-CPD (both 1:1000 CosmoBio). Secondary Abs used include Rhodamine Red-X conjugated AffiniPure Goat anti-rabbit or anti-mouse IgG and fluorescein (FITC)-conjugated AffiniPure Goat anti-rabbit IgG (Jackson Immuno-research Laboratories Inc). Coverslips were mounted on slides using

Vectashield[®] mounting media with DAPI (Vecta laboratories, Inc) and analyzed on a fluorescence microscope (Leica DM 5500B) with a Qimaging Retiga 2000R Fast 1394 camera. For each experimental time point ≥ 400 DAPI-positive cells (≥ 1200 in triplicate) were analyzed using Q-Capture Pro line intensity profile software with the intensity gated at ≥ 0.1 for positive LUDs for 6-4 PP or CPD staining. The accumulation of a protein at an LUD was considered positive if its' intensity was 10-fold greater than the line drawn over the rest of the nucleus. Positive LUDs were scored separately for each variable, either protein or UV induced lesion, and then divided by the number of cells analyzed using DAPI stain.

Mutation Frequency Assays

The HPRT assay was performed in A549 cells as described ³⁴ with the following modifications. After culturing cells for 1 week in media containing hypoxanthine, aminopterin, and thymidine (HAT selection) to eliminate background *HPRT* mutations, cells were stably depleted of FANCI with two unique shRNA targets versus a non-silencing control and selected with hygromycin. UV-induced HPRT mutants were obtained by seeding 6 plates at a confluence of 1×10^6 cells/10 cm dish 24 h prior to either mock treatment, 5, or 10 J/m² UV irradiation in a 254 nm SpectrolinkerTM XL-1500 (Dot Scientific, Inc). Post-treatment cells were allowed to recover to 6×10^6 cells or with mock cells 6 population doublings and 6×10^6 cells were seeded at a confluence of 1×10^6 /10 cm dish in media containing 24

uM 6-Thioguanine (6-TG) to select for HPRT-inactivated colonies. At the same time 200 cells were also seeded in 6-TG-free media to determine colony-forming efficiency. The frequency of inactivating mutations at the *HPRT* locus was calculated as the $[(\# \text{ of total 6-TG resistant colonies}) / (6 \times 10^6 \text{ cells seeded})] \times \text{the colony-forming efficiency}$. HPRT inactivation frequency represents the mean of three independent experiments. Individual colonies were picked and grown until enough cells were obtained for RNA isolation using TRIzol[®] Reagent (Life Technologies) according to the manufacturer's protocol. The HPRT gene was subjected to RT-PCR using SuperScript[®] (Invitrogen) followed by sequencing using overlapping primers HPRT1 5'CTTCCTCCTCCTGAGCAGTC3'; HPRT2–5'AAGCAGATGGC-CACAGAACT3'; HPRT3–5'CCTGGCGTCGTGATTAGTG3'; HPRT4–5'TTTAC-TGGCGATGTCAATAGGA3'; HPRT5–5'GACCAGTCAACAGGGGACAT3'; and HPRT6 5'ATGTCCCCTGTTGACTGGTC3'. Patient-derived *FA-J* cells were complemented with empty vector or FANCI^{WT} and treated as described with A549 cells. As *FA-J* cells do not make colonies, the % increase in 10uM 6-TG survival was calculated as the % of UV-irradiated cells surviving 6-TG minus the % of untreated cells surviving 6-TG. The % increase in 6-TG survival represents the mean of three independent experiments.

EdU Labeling

EdU incorporation was performed as described previously (Limsirichaikul et al 2009), except cells were seeded on coverslips and left untreated or UV-irradiated through 5 micron filters prior to 3 h incubation in 10uM EdU diluted in serum free media. When using global UV-irradiation, cells were left untreated and pulsed 45 min in 10 uM EdU or UV-irradiated and pulsed 16 h later for 45 min with 10 uM EdU. Cells were processed by Click-iT[®] EdU imaging kit (Invitrogen) using the manufacturer's instructions immediately followed by the above immunofluorescence protocol.

Western Blot

Cells were harvested and lysed in 150 mM NETN lysis buffer (20mM Tris (pH 8.0), 150 mM NaCl, 1mM EDTA, 0.5% NP-40, 1mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail) for 30 minutes on ice. Cell extracts were clarified by centrifugation at 14,000 rpm, protein was quantified by Bradford assay, and lysates were boiled in SDS loading buffer. Chromatin extracts were prepared as described ³⁵. For CPD immune-precipitation cells were lysed in 150 nM NETN buffer and sonicated. The lysate was quantified by Bradford assay and then pre-cleared with Protein A beads and immuno-precipitated overnight with CPD Abs. Proteins were separated by SDS-PAGE on 4-12% bis Tris or 3-8% Tris Acetate gels (Novex, Life Technologies) and electrotransferred onto nitrocellulose membranes. Membranes were blocked in 5% milk diluted in PBS. Antibodies used for Western blot analysis included anti-FANCI (1:1000 Sigma,

1:1000 E67 (previously described ³⁶, anti-Bactin (1:5000 Sigma), anti-MLH1 (1:500 BD Bioscience), anti-MSH2 (1:500 Calbiochem), anti-MSH2 (mouse specific, Santa Cruz), anti-XPF (1:1000 Neomarkers), anti-ERCC1 (1:500 Santa Cruz), anti-XPC (1:1000 Abcam), anti-CHK1 (1:500 Bethyl), anti-p317 CHK1 (1:500 Bethyl), anti-RPA32 (1:500 Bethyl), and anti-pS4/S8 RPA32 (1:500 Bethyl). Membranes were washed and incubated with horseradish peroxidase-linked secondary antibodies (Amersham 1:5000), and detected by chemiluminescence (Amersham). The ratio of phospho-protein to total protein was measured and quantified using Image J software.

FACS Analysis

FA-J cells cultured to ~ 80% confluency were left untreated or globally irradiated with 5 J/m² before collecting and fixing in 70% ethanol 4 h post UV irradiation. For antibody labeling, cells were rinsed with 1x PBS, permeabilized with 0.5% Triton-X 100 in PBS 20 minutes at room temperature, and then washed with 1% BSA/0.25% Tween-20 in PBS (PBS-TB) before re-suspending 1 h in PBS-TB with pS4/S8 RPA 32 antibody (1:250 Bethyl). Cells were then collected and washed 2x in PBS-TB prior to 1 h incubation in PBS-TB containing FITC-conjugated AffiniPure Goat Anti-Rabbit IgG (1:200 Jackson Immuno-research Laboratories, Inc.). After washing with PBS, cells were re-suspended in RNase A solution (100 µg/mL in PBS) for 20 min at room temperature and again washed with PBS prior to FACS analysis. Cells were labeled with propidium iodide prior

to analysis on a FACSCaliber flow cytometer (Becton-Dickinson) performed at the University of Massachusetts Medical School flow cytometry core facility using Cellquest software. The fluorescence intensity of pS4/S8 RPA 32 positive cells was gated as FITC-positive cell populations compared to no antibody control.

Statistics

All quantitative data were collected from experiments performed in triplicate, and expressed as mean \pm s.d. Differences between experimental groups were calculated using a two-tailed, unpaired student t-test using Excel Microsoft or Graphpad. Where shown, asterisks denote significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$.

CHAPTER III:

CISPLATIN RESISTANCE SCREEN IN BRCA2 MUTANT CELLS

Preface

The work in this chapter stems from a collaboration with Sharon Cantor and Michael Green's laboratories. Michael's former graduate student Ryan Serra was essential for helping me launch the shRNA screen. Ryan taught me the importance of optimization prior to launching the screen and helped me organize the validation scheme after recovering the candidates. His guidance and constructive criticism was ongoing throughout this project and has been a tremendous help. Amy Virbasius aided in the preparation of virus for candidate validation. Although I proceeded with the validation process of the candidates, several undergraduates and high school students assisted in the counting of colonies so that it could be performed in a blind format.

A former rotation student, Janelle Hayes, assisted with depletion of additional NuRD complex members and also helped to raise virus used to deplete CHD4 in other various experiments. Panagiotis Konstantinopoulos from Dana Farber compiled the mutations from BRCA2 mutant ovarian tumors and performed the Kaplan Meier plot analysis to examine progression free survival and overall survival in tumors with different levels of CHD4 mRNA expression.

Abstract

Therapies taking advantage of underlying defects in DNA repair are effective on cancer cells harboring mutations in the hereditary breast cancer genes, *BRCA1* or *BRCA2*. As with traditional chemotherapies however, resistance develops. One mechanism includes reversion mutations in *BRCA1* or *BRCA2* that restore functional proteins and RAD51-based homologous recombination. More perplexing is the development of resistance without reversion mutations. Here, I find, in a genome-wide shRNA screen, that depletion of the chromatin-remodeling factor CHD4 confers cisplatin resistance in *BRCA2*-mutant cells. Rescue by CHD4 depletion does not improve RAD51 foci formation as compared to rescue by *BRCA2* reversion mutation. However rescue similarly depends on *BRCA2* suggesting that CHD4 depletion creates a gain-of-function for the N-terminal truncated *BRCA2* protein. Consistent with CHD4 modulating therapeutic response, low CHD4 expression correlates with cisplatin resistance in *BRCA2*-mutant cell clones and poor patient response/survival in *BRCA2*-mutant ovarian tumors.

Introduction

Fanconi anemia (FA) and hereditary breast and ovarian cancer derive from common underlying gene defects that compromise DNA repair by homologous recombination (HR). Thus, FA patient cells and BRCA-associated tumors are sensitive to DNA damaging agents that require homologous recombination (HR) for repair processing. Loss of HR in BRCA1 and BRCA2-mutant cancer cells also elicits synthetic lethality with PARP1 inhibitors (PARPi). As such, cisplatin and PARPi alone or in combination are actively being tested on a diverse set of tumors with suspected *BRCA* or HR associated pathway mutations (Rouleau et al. 2010; Ang et al. 2013).

The efficacy of these therapies is limited, however by the development of resistance. One mechanism underlying resistance in *BRCA* cancers is re-established DNA repair. In particular, HR is restored in both BRCA1 and BRCA2 cancer cells through genetic rewiring events that re-instate functional gene products (Sakai et al. 2008; Swisher et al. 2008; Edwards et al. 2008; Sakai et al. 2009). Alternatively, in BRCA1 mutant cells loss of the DNA repair protein 53BP1 can restore DNA end resection and reinstate HR (Cao et al. 2009; Bunting et al. 2010; Bouwman et al. 2010). More importantly, increased 53BP1 expression levels correlate with improved progression free survival and overall survival in triple negative breast cancer patients (Bouwman et al. 2010). In BRCA2 mutant cells, 53BP1 depletion is not sufficient to restore HR as BRCA2 is thought to

function downstream of DNA end resection (Bouwman et al. 2010).

Interestingly, *BRCA2* reversion mutations and genetic studies have uncovered essential domains required for HR. *BRCA2* has 8 BRC repeats that are important for binding and loading the recombinase RAD51 onto resected DNA ends (Jensen 2013). Surprisingly, only two BRC repeats are required for HR as long as the *BRCA2* C-terminal DNA binding domain and the RAD51-DNA filament binding domains are also intact (Siaud et al. 2011). *BRCA2* mutant cancer cells and cells from Fanconi Anemia FA-D1 patients are defective for repair by HR, correspondingly, these *BRCA2* mutations are predicted to truncate the protein (Howlett et al. 2002; Sakai et al. 2009). Whereas promoter methylation and loss of *BRCA1* expression occurs in triple negative breast cancer, this is not the case in *BRCA2* mutant cancers (Collins et al. 1997; Matros et al. 2005). Additionally, *BRCA2* nullizygous mice result in embryonic lethality, whereas truncated forms of *BRCA2* are sufficient for survival (Ludwig et al. 1997; Connor et al. 1997). Taken together, these findings suggest that the N-terminal *BRCA2* region could have HR independent functions required for cell viability.

An interesting and perplexing finding is that *BRCA2* mutant cells can develop resistance without reversion mutations or signs of restored HR. *BRCA2* mutant cancer cell clones that are resistant to either cisplatin or PARP inhibition have been described (Sakai et al. 2008; Sakai et al. 2009; Norquist et al. 2011).

Moreover, chemoresistance in BRCA2 mutant cancers occurs without *BRCA2* reversion mutations in about half of ovarian tumors (Norquist et al. 2011). Thus, in BRCA2 mutant cancer cells, the absence of RAD51 foci does not accurately predict therapy response and the means by which tumors become resistant to therapy remains to be determined.

In an attempt to uncover how BRCA2 mutant cancer cells gain resistance to therapy independently of genetic reversion I performed a genome-wide RNA interference screen in BRCA2 mutant ovarian cancer cells to identify genes required for cellular sensitivity to cisplatin. The most robust candidate identified from my screen was the chromodomain helicase, CHD4, a member of the chromatin remodeling complex NuRD (Lai & Wade 2011). CHD4 depletion in several BRCA2 mutant cell lines conferred resistance to cisplatin. More importantly, examination of previously described BRCA2 mutant cell lines that were defective for HR and resistant to cisplatin for unknown reasons revealed that these clones have lost expression of CHD4 (Sakai et al. 2009). Given that de novo loss of CHD4 could propel resistance to cisplatin in a BRCA2 mutant background in cell culture, we further examined if CHD4 expression levels could predict patient response in BRCA2 mutant ovarian tumors in the clinic. Astoundingly, low CHD4 expression levels significantly correlated with poor progression free survival and reduced overall survival in BRCA2-mutant ovarian tumors.

Results

Genome wide screen identifies CHD4 as a modulator of cisplatin response in BRCA2 mutant cells

To identify genetic determinants of cisplatin resistance in BRCA2 mutant cells, we employed a survival-based, loss of function RNAi screen. The screen was performed in PEO1 cells that originate from a high-grade serous ovarian tumor that lacks wild-type BRCA2, but carries an allele with a c.617delT frameshift mutation (Sakai et al. 2009). In contrast with the wild-type 3,418-residue protein, the mutant allele encodes a truncated BRCA2 protein of 2,002 amino acids that lacks the C-terminus and 4 BRC repeats (Figure 3-1). Consequently, the mutant protein fails to efficiently promote RAD51-based HR and PEO1 cells are sensitive to cisplatin (Figure 3-1). Similar to other *BRCA* mutant cell lines, cisplatin resistance in PEO1 cells can develop through unknown mechanisms or by the acquisition of reversion mutations that restore BRCA2 sequences critical for HR (Sakai et al. 2009). The cisplatin resistant PEO1 derived clone C4-2 expresses a revertant, full length protein that migrates slower and more similar to the wild-type BRCA2 protein (390 kDa) as compared to the parental truncated protein (224 kDa) (Figure 3-1). As expected the functional BRCA2 restores robust RAD51 foci that are absent in parental PEO1 cells despite cisplatin-induced DNA damage detected by γ H2A.X foci (Figure 3-1). Reinstated BRCA2 enhances resistance to cisplatin, as well as DNA double

strand break-inducing agents, such as zeocin (Figure 3-1).

For the genome-wide shRNA screen, cisplatin sensitive PEO1 cells were infected with viral pools containing the pGIPZ library. Cells containing shRNA vectors were selected with puromycin, cisplatin treated and analyzed for colony formation (see Methods, Figure 3-2). The most pronounced increase in colony formation after cisplatin was observed with depletion of the chromodomain helicase, CHD4 a member of the nucleosome remodeling and deacetylase (NuRD) complex (Tong et al. 1998; Wade et al. 1998; Zhang et al. 1998). Four shRNAs directed against CHD4 showed this effect, making off-target effects unlikely (Figure 3-3). Depletion of a distinct NuRD-complex protein, MBD2, in PEO1 cells reduced cellular fitness and enhanced cisplatin sensitivity (Figure 3-4). This finding suggests that CHD4 depletion and not loss of the NuRD remodeling complex rescues PEO1 cells. Moreover, given that CHD4 depletion increased cisplatin resistance in two additional BRCA2 mutant cell lines, Fanconi anemia (FA) cell line, FA-D1 and in the pancreatic cancer cell line, CAPAN-1, these findings are not specific to a distinct cell line (Figure 3-5).

Analysis of cell cycle profiles in response to cisplatin

We next examined cell cycle analysis in response to cisplatin in PEO1 cells depleted of CHD4 verses a non-silencing control (NSC) and compared this C4-2 cells expressing a NSC (Figure 3-6). Cell cycle analysis revealed that CHD4 depleted PEO1 cells more closely resembled the profile of BRCA2

proficient cells. Specifically, while the majority of cells were all arrested in G2 phase within 48 hours after cisplatin treatment, only the BRCA2 restored C4-2 cells and CHD4 depleted PEO1 cells were able to recover 60 hours after the insult and proceed into the G1 phase. At this dose of cisplatin, PEO1 cells containing the NSC remain stuck in G2 phase or develop sub-G1 content suggesting the accumulation of apoptotic events 120 hours after the insult. Conversely, the cell cycle profiles of BRCA2 restored C4-2 cells and CHD4 depleted PEO1 cells are identical to the profiles of their untreated counterparts by 120 hours after cisplatin treatment indicating that their cell cycle is fully recovered (Figure 3-6).

CHD4 depletion in BRCA2 mutant PEO1 cells does not restore HR repair

The BRCA2 N-terminal mutant in PEO1 cells is defective for HR due to missing functional domains. However, given the cisplatin resistance phenotype associated with CHD4 depletion in PEO1 cells, we considered that these domain requirements could be circumvented by CHD4 depletion. CHD4 depletion could instead propel *BRCA2* reversion mutations and restore HR. Thus, we addressed if CHD4 depletion in PEO1 cells restored RAD51 foci. As expected, we observed cisplatin-induced RAD51 foci co-incident with γ H2A.X in the BRCA2 revertant C4-2 cells, but not in the parental PEO1 cells that express CHD4 (Figure 3-7). These RAD51 foci did not measurably improve in CHD4 depleted PEO1 cells although γ H2A.X foci were clearly induced following cisplatin (Figure 3-7).

Moreover, examination of chromatin bound RAD51 in unperturbed and cisplatin treated PEO1 cells also suggested that CHD4 depletion did not enhance, but rather slightly reduced RAD51 localization to the nucleus (Figure 3-8). Furthermore, CHD4 depletion did not alter the expression or migration of the BRCA2-mutant species suggesting that the mutant species did not undergo a genetic reversion to restore the full length protein (Figure 3-8). Notably, CHD4 expression and chromatin localization was not dependent on the BRCA2 C-terminal domain given the similar chromatin association of CHD4 in PEO1 and C4-2 cells (Figure 3-8). Collectively, these findings suggest that CHD4 depletion in PEO1 cells confers cisplatin resistance without restoration of BRCA2 function in HR.

Toxicities associated with CHD4 depletion are ameliorated in BRCA2 mutant cells

Enhanced cisplatin resistance by CHD4 depletion was counterintuitive given reports that CHD4 depletion causes sensitivity to DNA damaging agents (Luo et al. 2000; Pegoraro et al. 2009; Smeenk et al. 2010; Sims & Wade 2011; Polo et al. 2010; Larsen et al. 2010; Urquhart et al. 2011). Thus, we examined CHD4 depletion in the BRCA2 revertant C4-2 cells following exposure to distinct DNA damaging agents. In support of a role in DNA repair, we found that CHD4 depletion in C4-2 cells enhanced sensitivity not only to cisplatin, but also to zeocin and the PARPi, Olaparib (Figures 3-9, 3-10). In contrast, CHD4 depletion

in PEO1 cells enhanced resistance to each of these agents, albeit not to the level obtained in C4-2 cells with restored BRCA2 function (Figures 3-3, 3-10). Consistent with other reports, even without DNA damage, CHD4 depletion reduced plating efficiency (Figure 3-11) and induced γ H2A.X (Figure 3-12) (Smeenk et al. 2010). However, while this effect was detected in C4-2 and U2OS cells, it was not detected in BRCA2 mutant PEO1 or FA-D1 cells (Figures 3-11, 3-12).

Cisplatin resistance mediated by CHD4 depletion is unique to BRCA2 mutant cells

We next sought to examine if CHD4 depletion uniquely rescued other HR defective cells. To test this idea, we analyzed the effect of CHD4 depletion in BRCA1 mutant breast cancer cell lines, HCC1937 and SUM1315MO2. CHD4 depletion as compared to control profoundly reduced proliferation and changed the morphology of cells such that too few cells were available for sensitivity assays (Figure 3-13). Correspondingly, immunofluorescence experiments revealed that CHD4 depletion enhanced γ H2A.X foci formation (Figure 3-14). We also examined CHD4 depletion in other HR defective cell lines including FANCD1-deficient (FA-J cells), FANCD2-deficient (PD20 cells), or XPF-deficient (XP-F cells). While colony-forming efficiency was not overtly affected, CHD4 depletion did not enhance cisplatin resistance as found in BRCA2-mutant cells (Figure 3-15).

CHD4 depleted PEO1 cells are uniquely sensitive to the histone deacetylase inhibitor Trichostatin A

Next, we analyzed if CHD4 depletion generated cross-resistance to other DNA damaging agents in BRCA2 mutant cells. However, CHD4 depletion in PEO1 cells did not enhance resistance to 6-thioguanine (6-TG) or the bifunctional alkylator melphalan (Figure 3-16). Because CHD4 functions in the NuRD complex with histone deacetylases, we also examined the effects of the histone deacetylase inhibitor, Trichostatin A (TSA) (Tong et al. 1998; Wade et al. 1998; Zhang et al. 1998; Lai & Wade 2011). Interestingly, CHD4 depletion in PEO1 cells, but not C4-2 cells, enhanced sensitivity to TSA (Figure 3-16). These results suggest that reduced CHD4 expression in BRCA2 mutant cells could bestow an altered chromatin state that could be targeted and effectively treated with histone deacetylase inhibitors.

CHD4 depletion does not promote cisplatin resistance in BRCA2 deficient cells

To further investigate the effect of CHD4 depletion in cells defective for BRCA2 function, we used RNAi to deplete BRCA2 alone or in combination with CHD4. As expected, BRCA2 depletion, similar to CHD4 depletion alone, induced γ H2A.X and apoptosis as measured by caspase3 cleavage in C4-2 (Figure 3-17) and U2OS cells (Figure 3-18). Notably, these outcomes were similar in CHD4 and BRCA2 co-depleted cells (Figures 3-17, 3-18). Furthermore, the cisplatin

sensitivity due to BRCA2 depletion was not alleviated by CHD4 co-depletion in either C4-2 or U2OS cells (Figures 3-17, 3-18). Together these findings suggest that CHD4 depletion does not rescue BRCA2-deficient cells.

To test the possibility that rescue requires the N-terminal mutant BRCA2 species present in BRCA2 mutant PEO1 cells (Figure 3-1), we again analyzed the effects of CHD4 and CHD4/BRCA2 co-depletion in PEO1 cells. We found that BRCA2 RNAi substantially reduced the expression of the N-terminal BRCA2-mutant product (Figure 3-19). Suggesting functional consequences for loss of the N-terminal BRCA2 mutant, γ H2A.X expression was enhanced upon BRCA2 depletion (Figure 3-19). To a lesser degree CHD4 and BRCA2 co-depletion also induced γ H2A.X expression that was not detected in PEO1 cells with CHD4 depletion alone (Figure 3-19). The cisplatin resistance obtained by CHD4 depletion in PEO1 cells was also suppressed by BRCA2 co-depletion (Figure 3-19). Collectively, these data argue that the N-terminal mutant BRCA2 species contributes to suppressing γ H2A.X induction and partially mediates cisplatin resistance in CHD4 depleted PEO1 cells.

Derived cisplatin resistant ovarian cancer cell clones correspond with de novo loss of CHD4 expression

Evidence suggests that N-terminal BRCA2 peptides that retain BRC repeats while defective in HR have partial function that promotes viability (Ludwig et al. 1997). PEO1 cells, similar to CAPAN-1 and FA-D1 cells, retain BRC

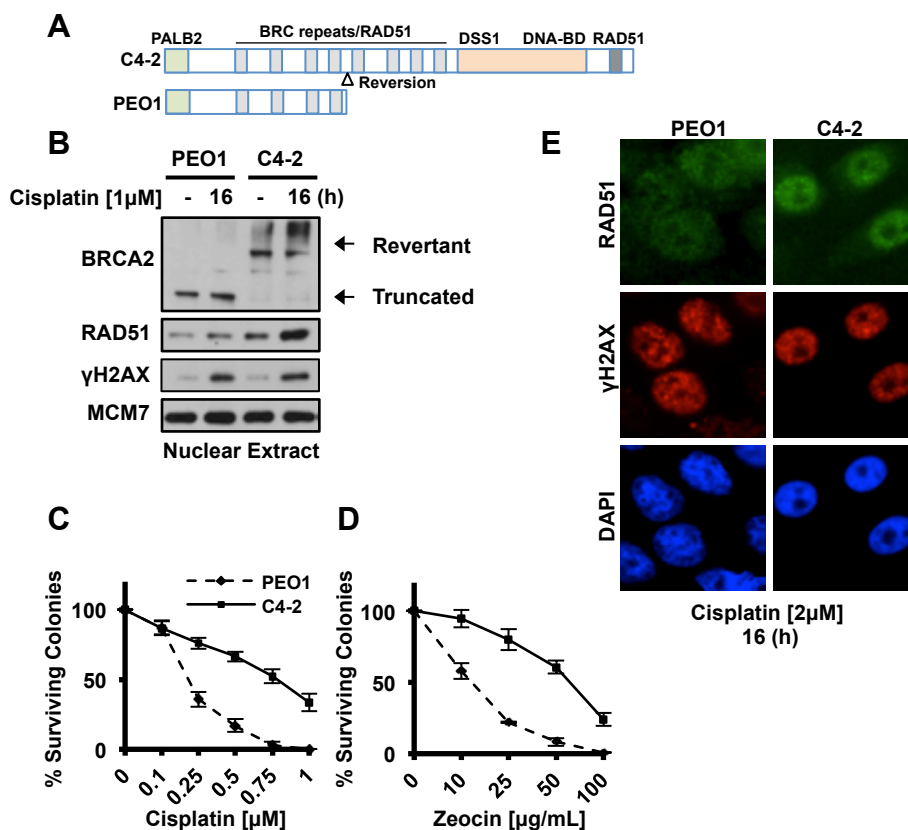
repeats (Howlett et al. 2002; Sakai et al. 2008; Sakai et al. 2009). Given that BRCA2 mutant cells typically retain some form of truncated BRCA2, we sought to determine if CHD4 loss had clinical relevance. To investigate if derived cisplatin resistance results from de novo CHD4 loss, we analyzed CHD4 expression in a series of previously derived PEO1 cisplatin resistant clones lacking BRCA2 reversion mutations and Rad51 focal induction (Figures 3-20, 3-21) (Sakai et al. 2009). As compared to the parental PEO1, or BRCA2 revertant clone C4-2, resistant clones, C4-4, C4-11, and C4-13 had reduced CHD4 protein expression levels (Figure 3-22). Interestingly, ectopic expression of GFP tagged CHD4 in the cisplatin resistant clone C4-4 was sufficient to restore sensitivity to cisplatin (Figure 3-23). Moreover, similar to CHD4 depleted PEO1 cells (Figure 3-16), we found that cisplatin resistant clones with low CHD4 expression were also sensitive to the HDAC inhibitor TSA as compared to parental PEO1 and revertant C4-2 cells (Figure 3-24).

CHD4 expression predicts patient response to therapy in BRCA2 mutant ovarian tumors

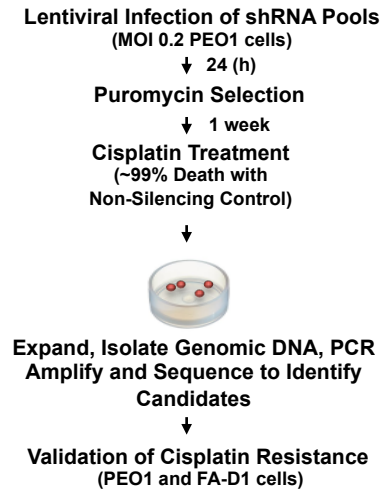
In light of our findings, we hypothesized that CHD4 expression levels could predict response to cisplatin based therapy specifically in BRCA2 mutant tumors. Thus, CHD4 mRNA expression was examined in 312 spontaneous serous ovarian tumors for which overall survival (OS) was documented and 262 ovarian tumors for which progression free survival (PFS) was documented using

database analysis. Median CHD4 expression was used as a threshold to classify tumors as CHD4 over-expressed (above median) or CHD4 under-expressed (below median) and PFS and OS were assessed in patients. In spontaneous tumors, CHD4 expression levels displayed no significant correlation with PFS or OS (Figure 3-25). Correlation between CHD4 expression and PFS in 30 BRCA1 mutant tumors or OS in 36 BRCA1 mutant tumors was also not significantly predictive of response (Figure 3-26). Compilation of BRCA2 mutant tumors revealed that the majority of tumors contain frameshift mutations or premature stop codons predicted to truncate the protein (Table 3-1). Strikingly, analysis of CHD4 expression in the BRCA2 mutant tumors revealed that CHD4 over-expression (above median) correlated with significantly improved PFS and improved OS in tumors from 32 patients (Figure 3-27). These results suggest that CHD4 mRNA expression levels could serve as a valuable predictor of response to chemotherapy in patient tumors carrying a BRCA2 mutation.

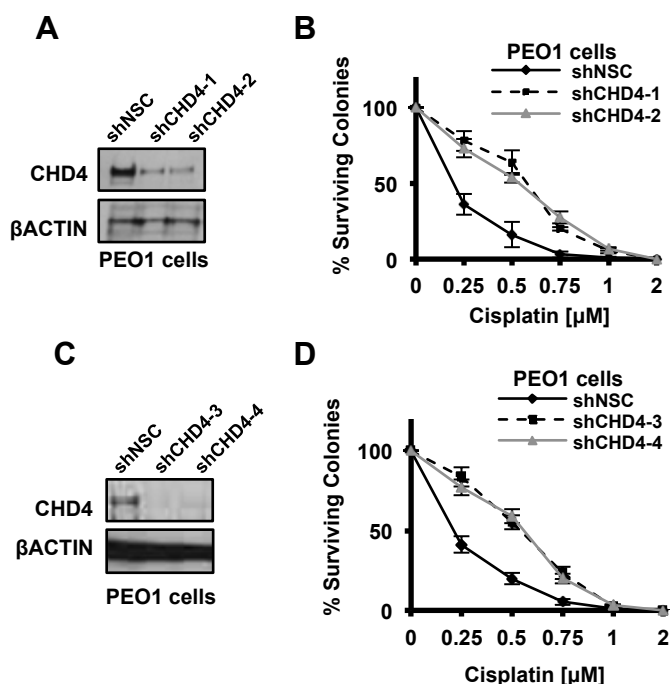
Figures



3-1 PEO1 cells express nuclear truncated BRCA2 and are sensitive to DNA damaging agents compared to BRCA2 revertant C4-2 cells (A) Cartoon showing BRCA2 mutant PEO1 and BRCA2 revertant C4-2 cells (B) Cells were left untreated or cisplatin treated and nuclear extracts were analyzed by immunoblot with the indicated Abs. (C) PEO1 and C4-2 cells were left untreated or Cisplatin treated and analyzed for colony survival. (D) PEO1 and C4-2 cells were treated as in C, except with Zeocin. (E) PEO1 and C4-2 cells were treated as in B and co-immunostained with the indicated Abs.

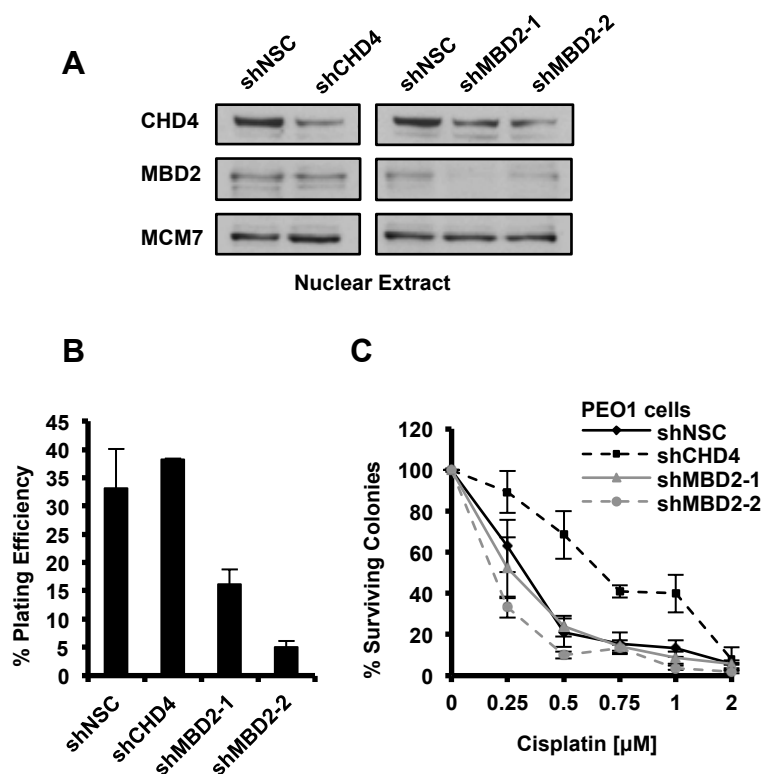


3-2 An RNA interference screen to identify mediators of cisplatin resistance in BRCA2 mutant cells Schematic of viral infection with shRNA pools and screening conditions.

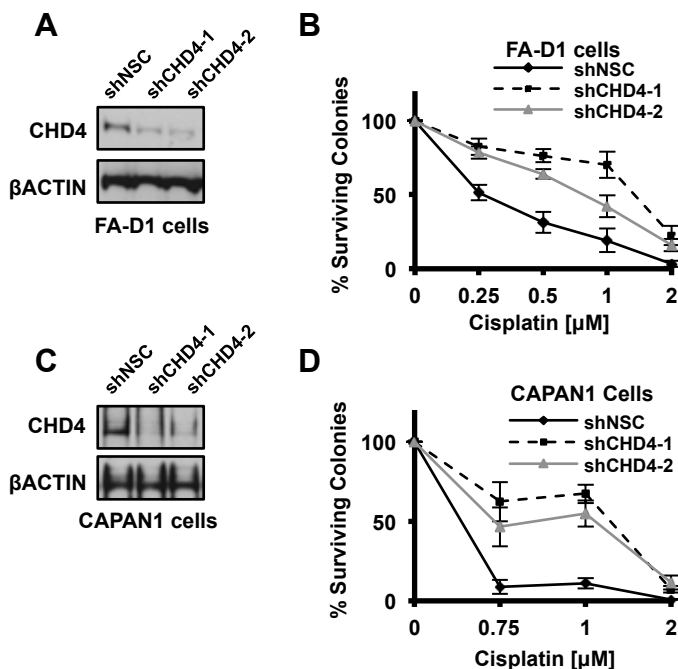


3-3 An RNA interference screen identifies CHD4 as a mediator of cisplatin resistance in BRCA2 mutant cells

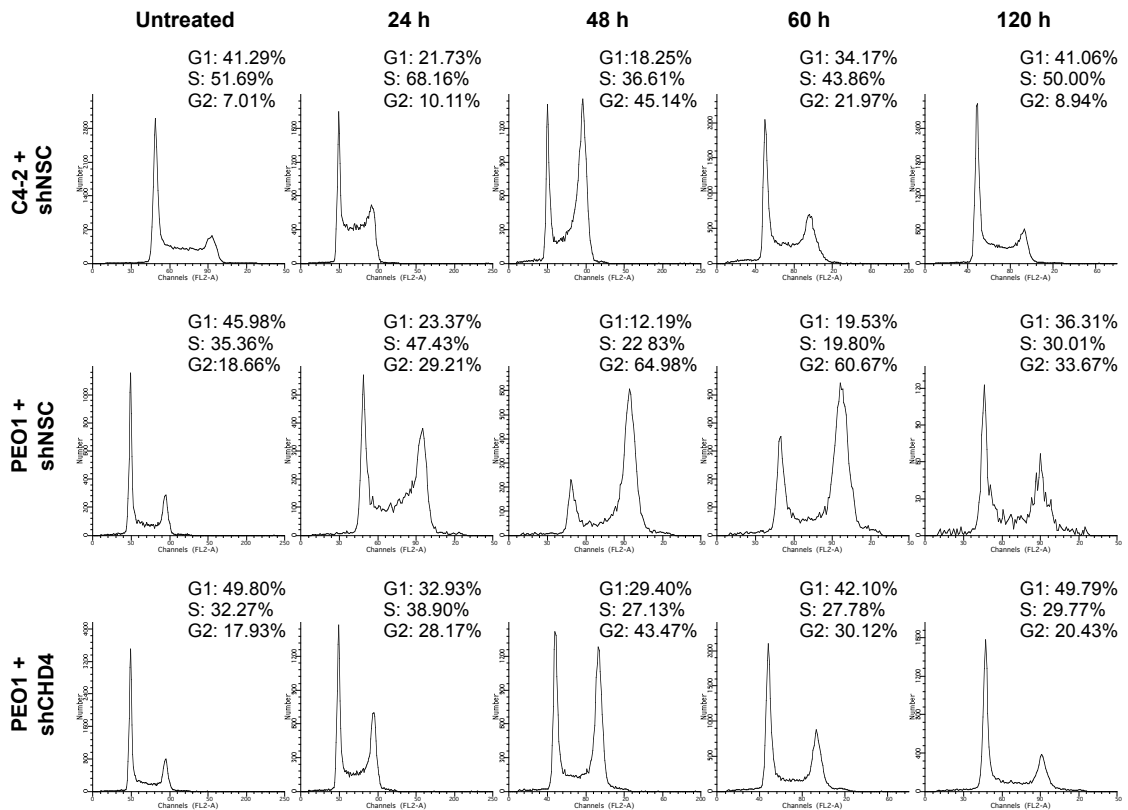
(A) PEO1 cells containing two unique pGIPZ shRNA vectors targeting CHD4 or non-silencing control (NSC) were analyzed by immunoblot and (B) were left untreated or Cisplatin treated and analyzed for colony survival. (C) PEO1 cells containing two unique pLKO.1 shRNA vectors targeting CHD4 or non-silencing control (NSC) were analyzed by immunoblot and (D) were left untreated or Cisplatin treated and analyzed for colony survival.



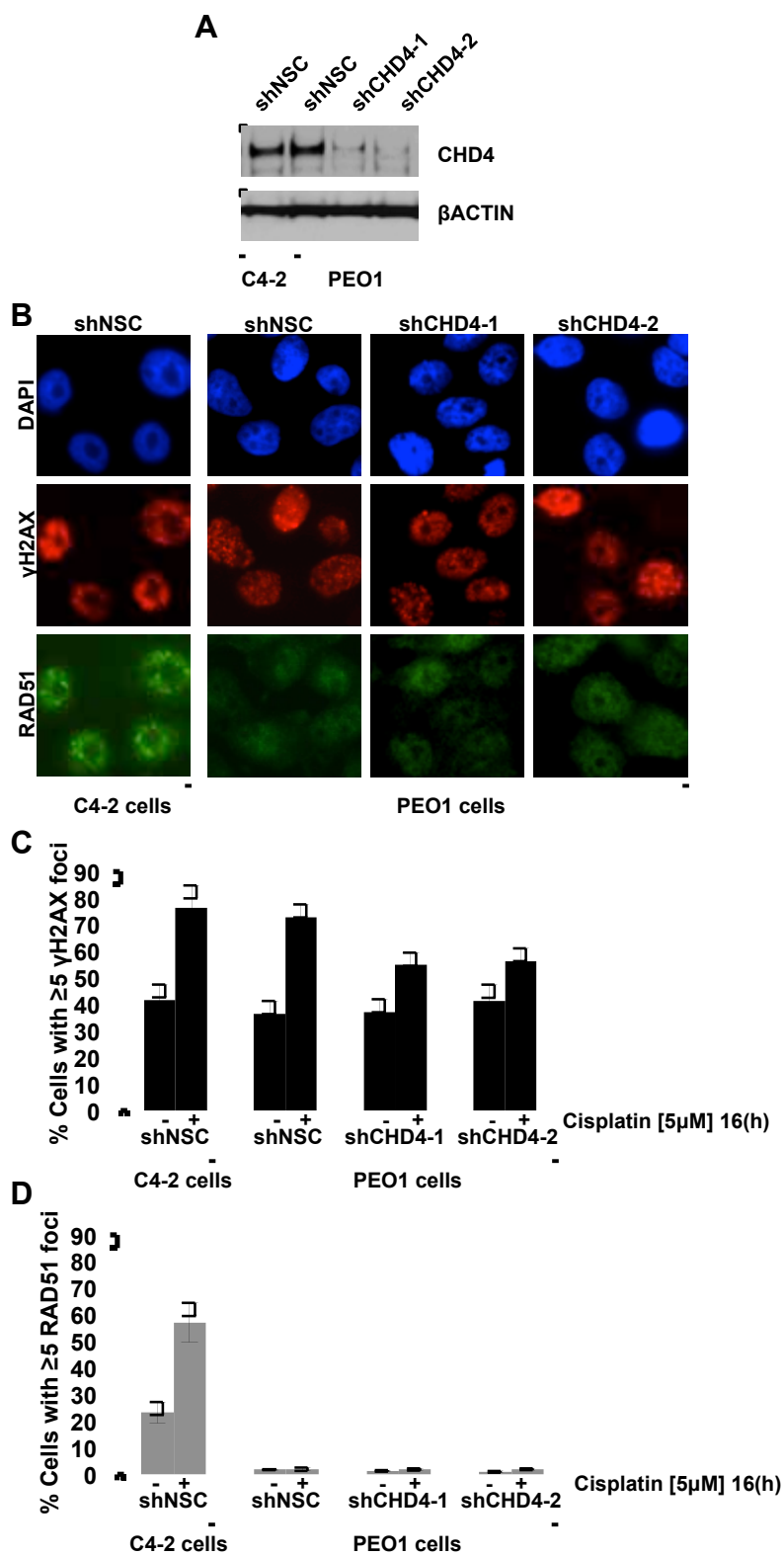
3-4 Depletion of the nucleosome remodeling (NuRD) complex member MBD2 does not mediate cisplatin resistance (A) PEO1 cells containing pLKO.1 shRNA vectors targeting NSC, CHD4, or two unique shRNA vectors targeting MBD2 were analyzed by immunoblot. (B) Cells were plated at low density and analyzed for colony forming efficiency. (C) Cells were left untreated or treated with cisplatin and quantified for colony survival.



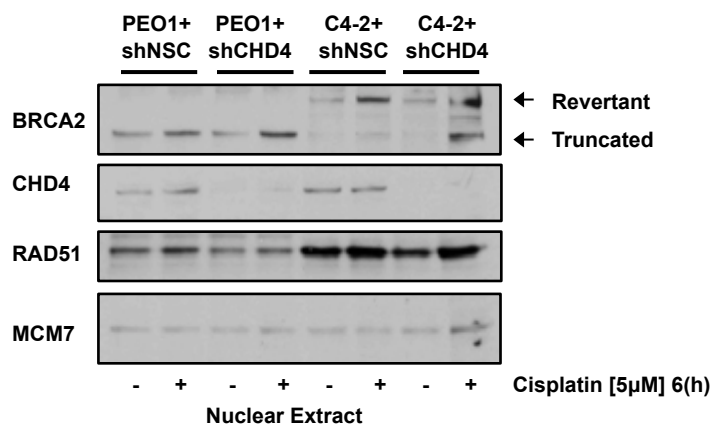
3-5 An RNA interference screen identifies CHD4 as a mediator of cisplatin resistance in BRCA2 mutant cells (A) FA-D1 Fanconi Anemia cells containing two unique pGIPZ shRNA vectors targeting CHD4 or non-silencing control (NSC) were analyzed by immunoblot and (B) were left untreated or Cisplatin treated and analyzed for colony survival. (C) CAPAN1 pancreatic cells containing two unique pGIPZ shRNA vectors targeting CHD4 or non-silencing control (NSC) were analyzed by immunoblot and (D) were left untreated or Cisplatin treated and analyzed for colony survival.



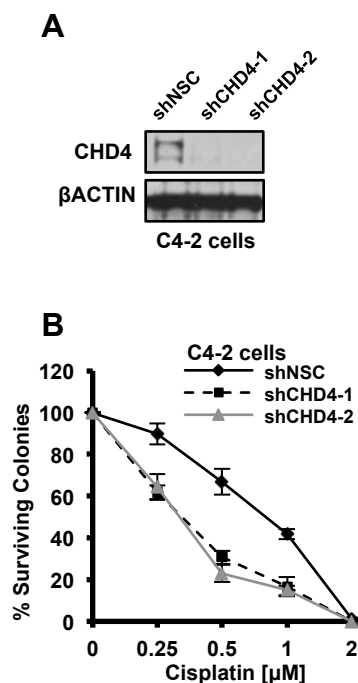
3-6 Cell cycle profiles of BRCA2 restored C4-2 cells and BRCA2 mutant PEO1 cells depleted of CHD4 versus control in response to cisplatin C4-2 cells expressing an shRNA directed against NSC or PEO1 cells expressing shRNAs directed against NSC or CHD4 were left untreated or Cisplatin treated for 24 h. Cells were collected at several time points, propidium iodide stained, and analyzed for cell cycle.



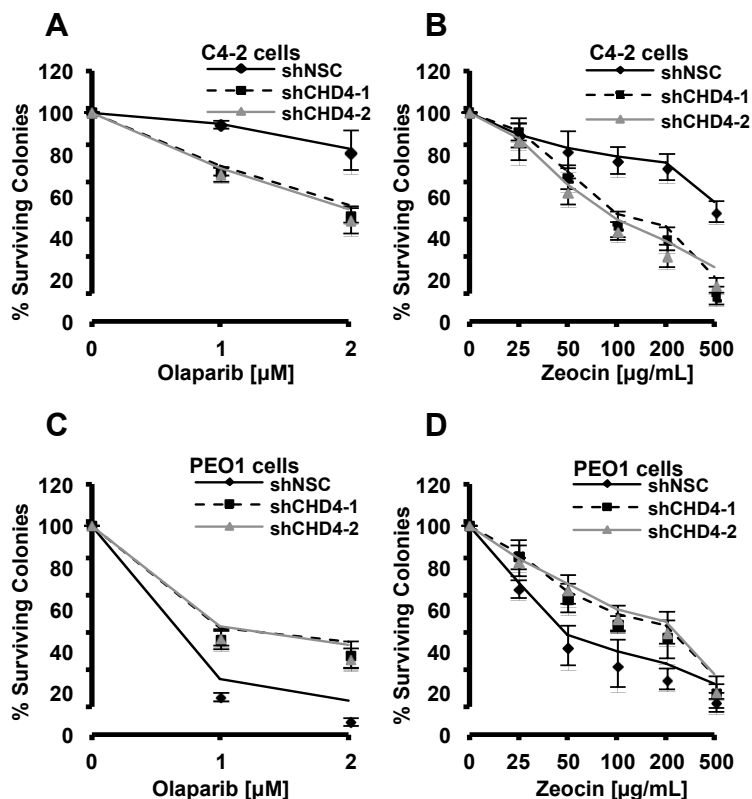
3-7 CHD4 depletion in PEO1 cells does not restore RAD51 foci formation (A) C4-2 and PEO1 cells containing pLKO.1 NSC vector or two unique shRNA vectors targeting CHD4 were analyzed by immunoblot with the indicated Abs. (B) Cells were left untreated or treated with 2 μ M Cisplatin for 16h and co-immunostained with the indicated Abs. A representative image of Cisplatin treated cells is shown. (C) quantification of % cells with ≥ 5 γ H2AX foci/cell or (D) % cells with ≥ 5 RAD51 foci/cell.



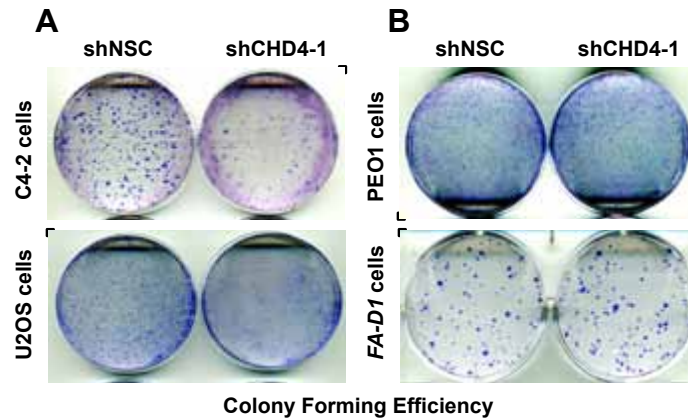
3-8 CHD4 depletion in PEO1 cells does not restore full length BRCA2 PEO1 and C4-2 cells expressing shRNAs directed against NSC or CHD4 were left untreated or Cisplatin treated and nuclear extracts were analyzed with the indicated Abs.



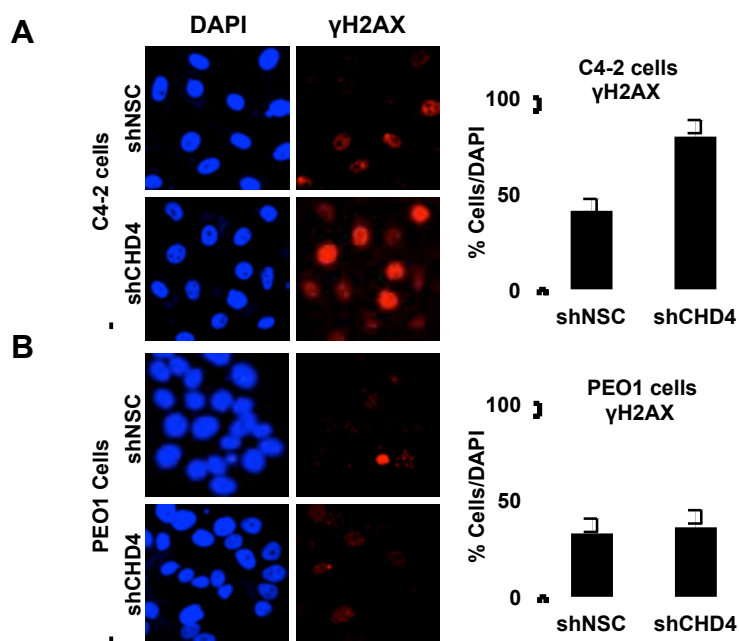
3-9 CHD4 depletion causes cisplatin sensitivity in BRCA2 proficient cells (A) BRCA2 revertant C4-2 cells containing two unique pGIPZ shRNA vectors targeting CHD4 or non-silencing control (NSC) were analyzed by immunoblot and (B) were left untreated or Cisplatin treated and analyzed for colony survival.



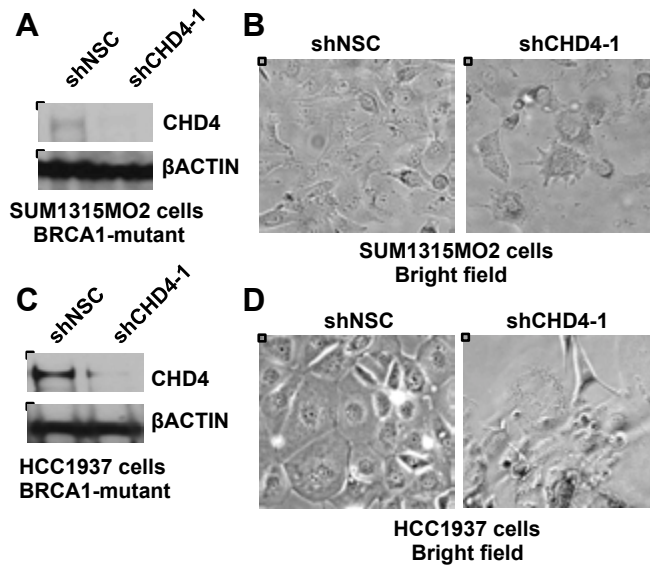
3-10 CHD4 depleted BRCA2 proficient cells are sensitive to DNA damage while BRCA2 mutant PEO1 cells are partially resistant (A) C4-2 cells containing pLKO.1 shRNA vectors targeting NSC or two unique vectors targeting CHD4 were plated at low density, left untreated or treated with the PARP inhibitor Olaparib, or (B) Zeocin, and quantified for colony survival. (C) PEO1 cells containing pLKO.1 shRNA vectors targeting NSC or two unique vectors targeting CHD4 were plated at low density, left untreated or treated with the PARP inhibitor Olaparib, or (D) Zeocin, and quantified for colony survival.



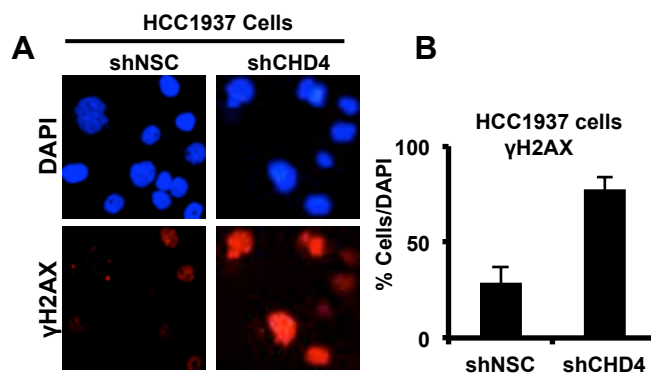
3-11 CHD4 depletion is toxic in BRCA2 proficient cells, yet uniquely tolerated in BRCA2 mutant cells (A) BRCA2 proficient C4-2 (top) and U2OS cells (bottom) containing pGIPZ shRNA vectors targeting CHD4 or non-silencing control (NSC) were seeded at low density and analyzed for colony forming efficiency after 7 days by fixation and Giemsa staining. (B) BRCA2 mutant PEO1 (top) and FA-D1 cells (bottom) were seeded at low density and analyzed for colony forming efficiency after 7 days by fixation and Giemsa staining.



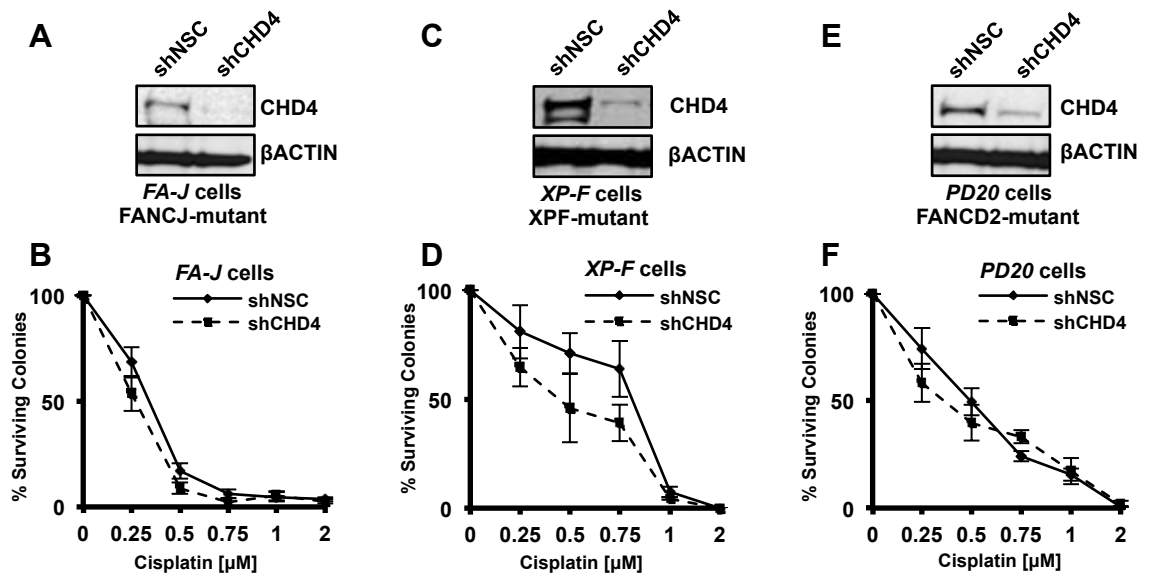
3-12 CHD4 depletion is toxic in BRCA2 proficient cells, yet uniquely tolerated in BRCA2 mutant cells (A) BRCA2 proficient C4-2 cells containing pLKO.1 shRNA vectors targeting CHD4 or NSC were immunostained with the indicated Abs and quantified for % cells with ≥ 5 H2AX foci (B) BRCA2 mutant PEO1 cells were treated as in A and quantified for % cells with ≥ 5 H2AX foci.



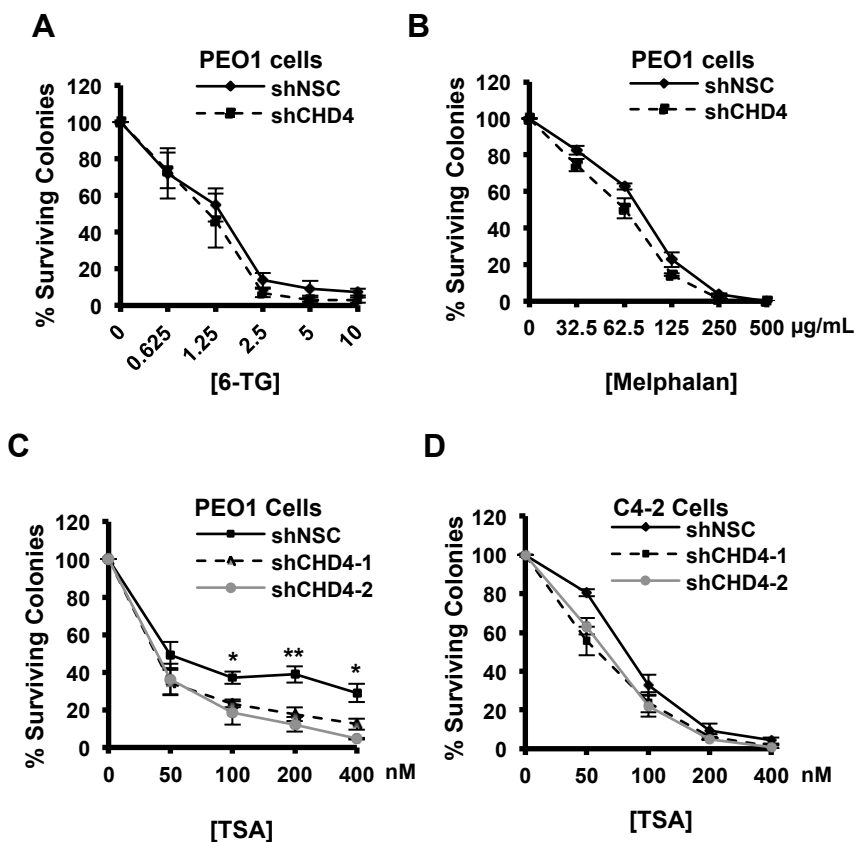
3-13 CHD4 depletion mediates toxicity in BRCA1 mutant cells (A) BRCA1 mutant SUM1315MO2 cells containing pLKO.1 shRNA vectors targeting CHD4 or NSC were analyzed by immunoblot and (B) representative bright field images are shown 4 days after puromycin selection. (C) BRCA1 mutant HCC1937 cells containing pLKO.1 shRNA vectors targeting CHD4 or NSC were analyzed by immunoblot and (D) representative bright field images are shown 4 days after puromycin selection.



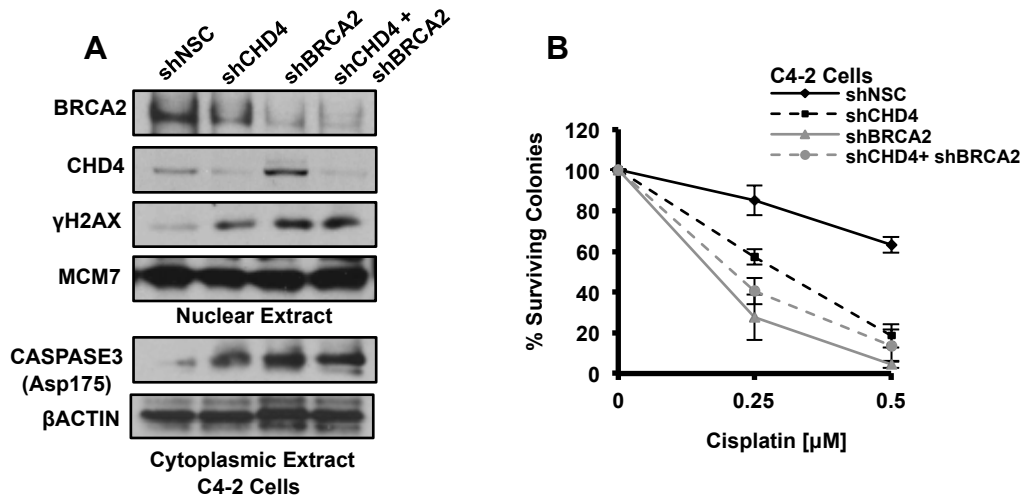
3-14 CHD4 depletion mediates toxicity in BRCA1 mutant cells (A) BRCA1 mutant HCC1937 cells containing pLKO.1 shRNA vectors targeting CHD4 or NSC were immunostained with the indicated Abs and (B) quantified for % cells with ≥ 5 H2AX foci.



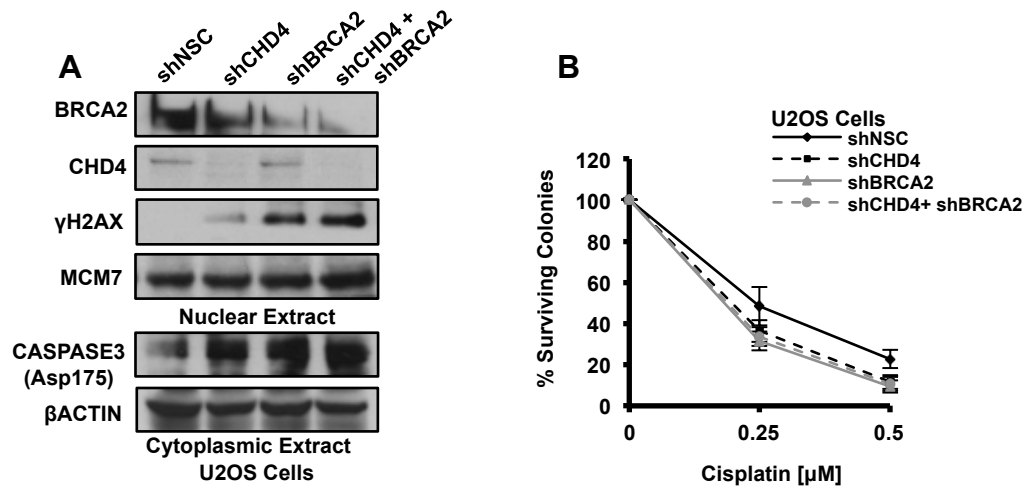
3-15 CHD4 depletion does not mediate cisplatin resistance in other DNA repair deficient cells (A) FA-J cells containing pLKO.1 shRNA vectors targeting CHD4 or NSC were analyzed by immunoblot and (B) left untreated or cisplatin treated and quantified for colony survival. (C) XP-F cells were treated as in A and (D) quantified as in B. (E) PD20 cells were treated as in A and (F) quantified as in B.



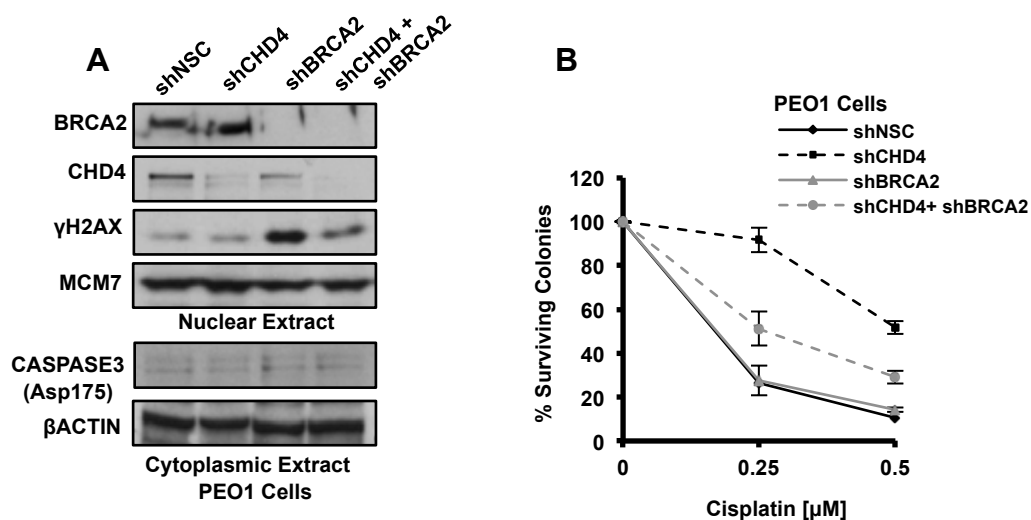
3-16 CHD4 depleted PEO1 cells are sensitive to the HDAC inhibitor Trichostatin A (TSA). (A) PEO1 cells containing pLKO.1 shRNA vectors targeting NSC or CHD4 were plated at low density, left untreated or treated with 6-Thioguanine (6-TG) or (B) Melphalan and analyzed for colony survival. (C) PEO1 cells or (D) C4-2 cells containing pLKO.1 shRNA vectors targeting NSC or two unique vectors targeting CHD4 were plated at low density, left untreated or treated with Trichostatin A and quantified for colony survival.



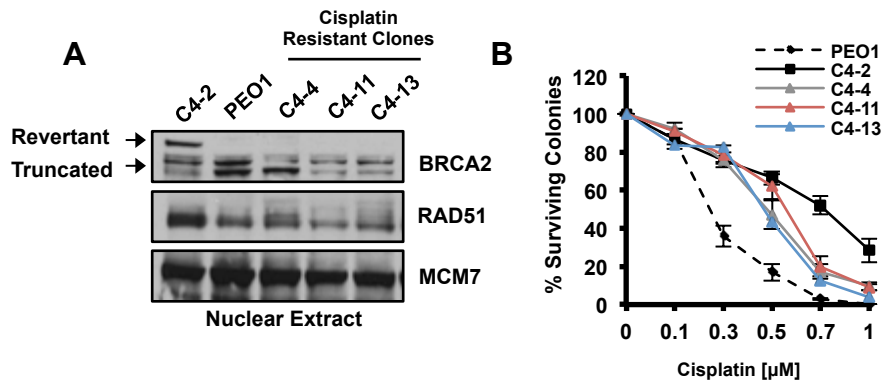
3-17 Co-depletion of CHD4 and BRCA2 in BRCA2 proficient C4-2 cells does not mediate cisplatin resistance (A) C4-2 cells were infected with pLKO.1 lentiviral vectors targeting NSC, CHD4, and BRCA2 or co-infected with vectors targeting CHD4 and BRCA2 and analyzed by immunoblot with the indicated Abs. (B) Cells were left untreated or treated with Cisplatin and analyzed for colony survival.



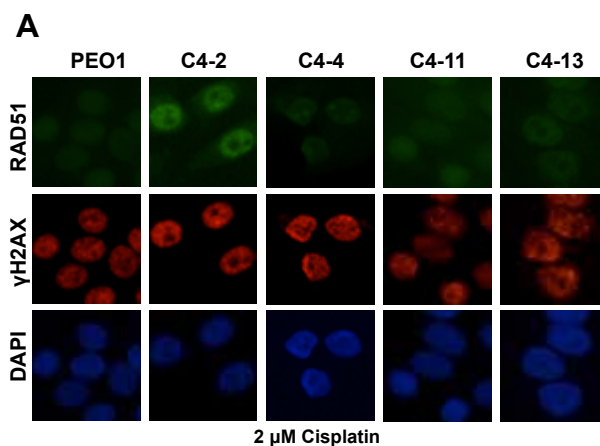
3-18 Co-depletion of CHD4 and BRCA2 in BRCA2 proficient U2OS cells does not mediate cisplatin resistance (A) U2OS cells were infected with pLKO.1 lentiviral vectors targeting NSC, CHD4, and BRCA2 or co-infected with vectors targeting CHD4 and BRCA2 and analyzed by immunoblot with the indicated Abs. (B) Cells were left untreated or treated with Cisplatin and analyzed for colony survival.



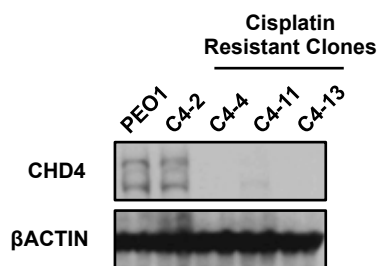
3-19 Cisplatin resistance mediated by CHD4 depletion in PEO1 cells requires expression of truncated BRCA2 (A) PEO1 cells were infected with pLKO.1 lentiviral vectors targeting NSC, CHD4, and BRCA2 or co-infected with vectors targeting CHD4 and BRCA2 and analyzed by immunoblot with the indicated Abs. (B) Cells were left untreated or treated with Cisplatin and analyzed for colony survival.



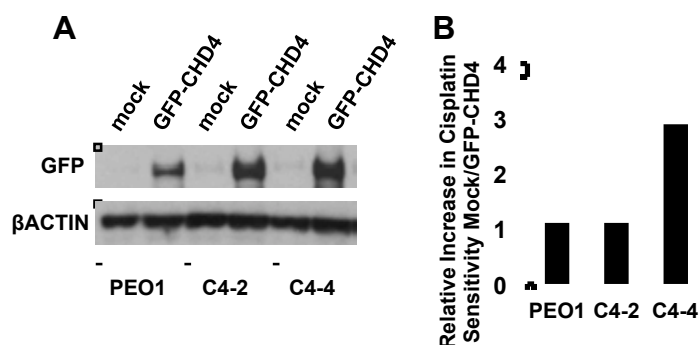
3-20 Cisplatin resistant clones retain truncated BRCA2 and gain resistance by an unknown mechanism (A) Nuclear extracts of BRCA2 revertant C4-2 cells, BRCA2 mutant PEO1 cells, and derived Cisplatin resistant clones C4-4, C4-11, and C4-13 were analyzed by immunoblot with the indicated Abs. and (B) left untreated or treated with cisplatin and analyzed for colony survival.



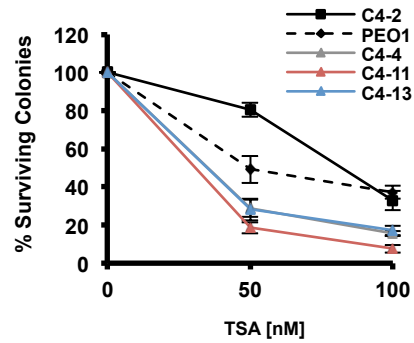
3-21 Cisplatin resistant clones do not restore RAD51 foci formation (A) BRCA2 mutant PEO1 cells, BRCA2 revertant C4-2 cells, and derived Cisplatin resistant clones C4-4, C4-11, and C4-13 were left untreated or treated with cisplatin and immunostained with the indicated Abs 16 hrs after treatment.



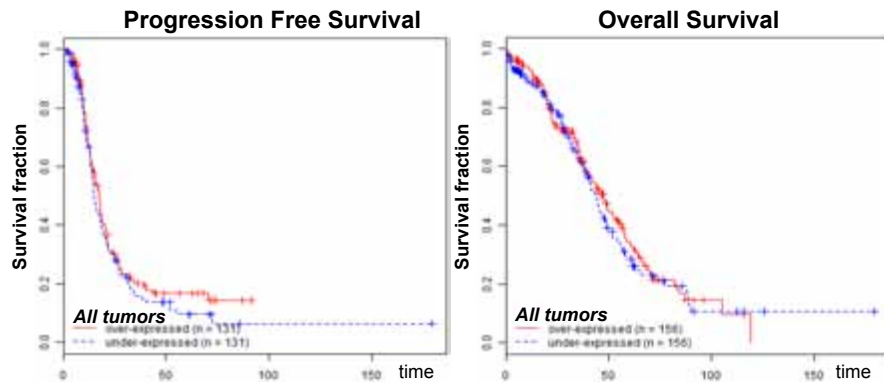
3-22 Cisplatin resistant clones have reduced CHD4 protein expression BRCA2 mutant PEO1 cells, BRCA2 revertant C4-2 cells, and derived cisplatin resistant clones C4-4, C4-11, and C4-13 were analyzed by immunoblot with the indicated Abs.



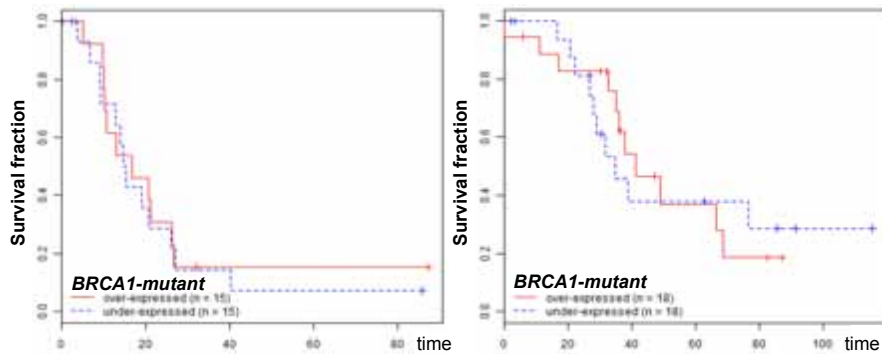
3-23 Ectopic expression of GFP-CHD4 restores sensitivity to C4-4 cells (A) PEO1, C4-2, and derived cisplatin resistant C4-4 cells were mock or GFP-CHD4 transfected and analyzed by immunoblot 36 hrs post transfection with the indicated Abs. (B) Cells were left untreated or treated with cisplatin and analyzed for colony survival. The relative increase in cisplatin sensitivity of mock transfected/GFP-CHD4 transfected cells is quantified.



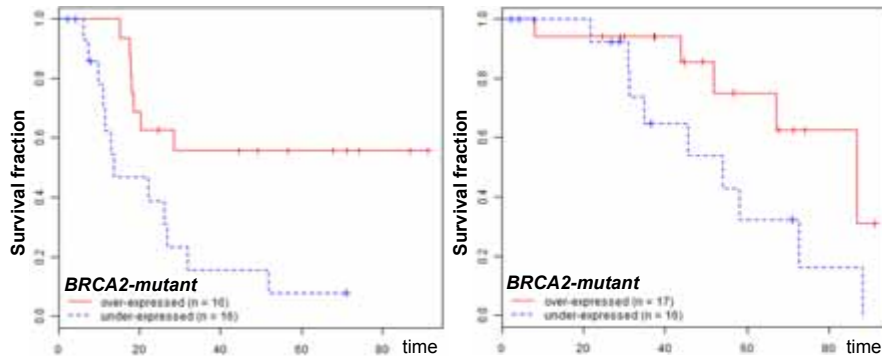
3-24 Cisplatin resistant clones are uniquely sensitive to Trichostatin A (TSA) BRCA2 revertant C4-2 cells, BRCA2 mutant PEO1 cells, and derived Cisplatin resistant clones C4-4, C4-11, and C4-13 were left untreated or treated with Trichostatin A (TSA) and analyzed for colony survival.



3-25 Progression Free Survival (PFS) and Overall Survival (OS) does not correlate with CHD4 mRNA expression levels in sporadic ovarian cancers CHD4 mRNA association with Progression free survival (PFS) ($p=0.313$) and Overall survival (OS) ($p=0.641$) in all serous ovarian tumors.



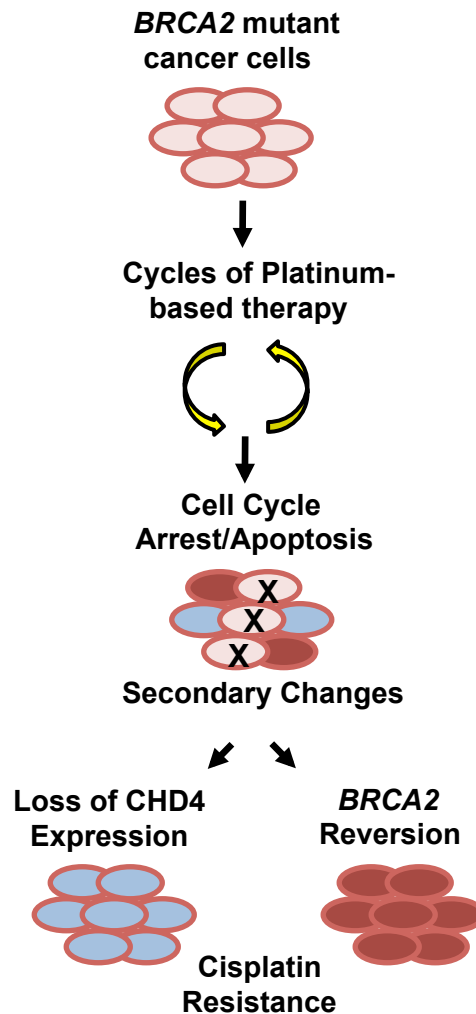
3-26 Progression Free Survival (PFS) and Overall Survival (OS) is not improved in BRCA1 mutant ovarian tumors with increased CHD4 mRNA expression CHD4 mRNA association with PFS ($p=0.715$) and OS ($p=0.916$) in BRCA1-mutant tumors.



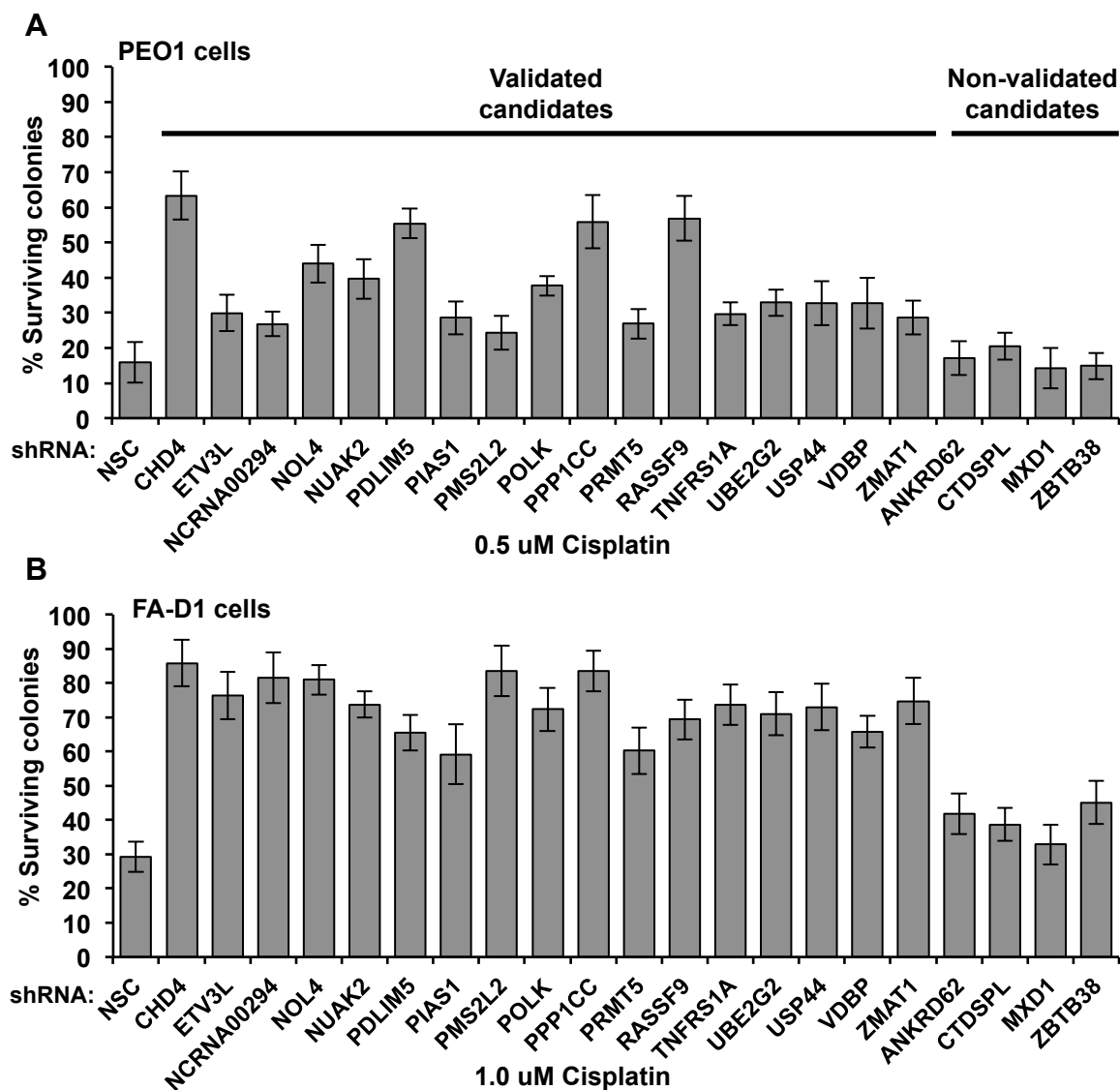
3-27 Progression Free Survival (PFS) and Overall Survival (OS) is improved in BRCA2 mutant ovarian tumors with increased CHD4 mRNA expression CHD4 mRNA association with PFS ($p=0.006$) and OS ($p=0.065$) in BRCA2-mutant tumors.

AA change	Mutation type
D1737fs	FS del
E1857fs	FS del
E342fs	FS del
K1406fs	FS del
L1491fs	FS del
L2696fs	FS del
L3277fs	FS del
Q1782fs	FS del
R1704fs	FS del
S1982fs	FS del
S1982fs	FS del
S1982fs	FS del
S1982fs	FS del
S1982fs	FS del
T219fs	FS del
T256fs	FS del
T2607fs	FS del
T3085fs	FS del
V1229fs	FS del
Y1710fs	FS del
G602fs	FS ins
E1143D	Missense
K1638E	Missense
T1354M	Missense
C711*	Nonsense
E294*	Nonsense
K3326*	Nonsense
K3326*	Nonsense
K3326*	Nonsense
R2394*	Nonsense
R2520*	Nonsense
S1882*	Nonsense
E2878_splice	Splice
V211_splice	Splice

Table 3-1 BRCA2 mutations in ovarian tumors from patients used for Kaplan-Meier estimation



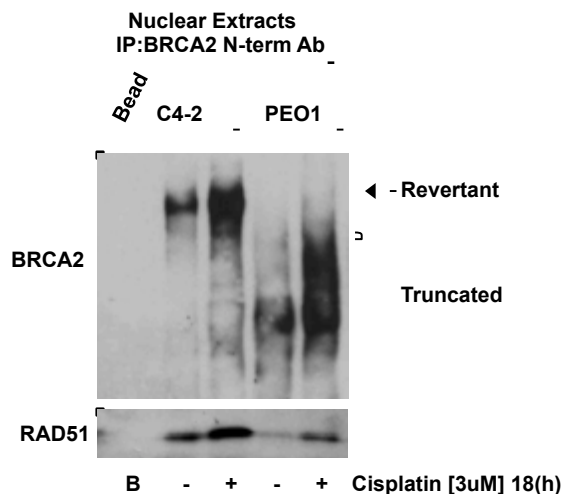
3-28 Model depicting how resistance to cisplatin occurs in *BRCA2* mutant cells. Upon cycles of treatment with cisplatin therapy resistant cells can emerge upon loss of CHD4 expression or genetic reversion of *BRCA2* to restore full length protein.



3-29 Partial validation of candidates identified in the shRNA screen (A) Candidates identified in the shRNA screen were depleted in PEO1 cells versus a non-silencing control (NSC) and examined for resistance to cisplatin at several concentrations, the 0.5 μ M concentration is shown. (B) FA-D1 cells were treated as in A, except the 1.0 μ M concentration is shown.

<u>DNA Metabolism</u>	<u>Transcription</u>	<u>Cell metabolism</u>	<u>Unknown</u>
AHNAK	ASXL2	ALDH2	ARMCX5
CHD4	ELL	ALG6	C1orf212
NAP1L2	ETV3	ATP11B	C9orf23
PMS2L2	ETV3L	CDO1	C10orf81
POLK	MXD1	CYP8B1	C20orf194
PRMT5	PIAS1	CRLS1	C21orf129
PRMT7	ZBTB38	DGKG	CXorf23
REV3L	ZNF24	DNAJA4	CCDC136
RNF20	ZNF280C	FECH	COMMD10
WDHD1	ZNF560	FMO9P	CTDSPL
	ZNF673	FUT9	EFCAB2
		GPX8	FAM26E
<u>RNA metabolism</u>	<u>Translational regulation</u>	HECTD1	KIAA0195
DDX1		HSD17B12	KIAA0355
DDX18	CPEB1	MAT1A	KIAA0494
DQX1	EIF4B	PSMC1	LRFN2
SFPQ	EIF4H	SUMF1	LRRC10B
		TMLHE	LRRC8B
<u>Signal transduction</u>	<u>Intracellular protein transport</u>	UBE2G2	MYO15B
ARHGEF17		VDBP	NCRNA00294
GPR64	BICD1		NOL4
GREB1	HSPA5	<u>Channels</u>	SHCBP1L
HTR3A	SLC15A4	KCNH1	SHISA9
NR4A2	SLC7A11	KCNH6	TMEM35
NUAK2	SLC04A1	KCNJ5	TTC38
PLCL1	SYTL5		UBE2D4
PPP2CB			WDR52
PTGES	<u>Vesicle trafficking</u>	<u>Immunity</u>	WDSUB1
RASSF9	ARCN10L	IL2RB	
SPA17	TPD52	LY6G6E	
TGFBR1		TLR5	
TNFRSF1A	<u>Cell motility/adhesion</u>		
<u>Cell cycle regulation</u>	ANKRD62		
ALMS1	COL28A1		
MKI67	CPNE3		
PPP1CC	MUC16		
USP44	PDLIM5		
	SCIN		

Table 3-2 List of candidates identified in the cisplatin resistance shRNA screen in BRCA2 mutant PEO1 cells based on gene ontology.



3-30 Truncated BRCA2 can interact with RAD51 in a cisplatin inducible manner C4-2 or PEO1 cells were left untreated or treated with cisplatin and collected at the indicated time point. Cells were lysed in CSK buffer and the cytoplasmic fraction was removed. The insoluble pellet was re-suspended in 150 nM NETN buffer and sonicated. The lysate was used for precipitation with either protein A beads alone or with an N-terminal BRCA2 ab.

Conclusions

By performing a genome-wide loss of function screen in BRCA2 mutant ovarian cancer cells, we have identified that loss of the NuRD chromatin-remodeling enzyme CHD4 promotes resistance to the chemotherapeutic agents cisplatin and partial resistance to the PARP inhibitor Olaparib (Figures 3-3, 3-10). CHD4 depletion in BRCA2 mutant cells promotes resistance to cisplatin independently of restored BRCA2 and canonical homologous recombination (Figures 3-7, 3-8, 3-28). CHD4 depletion in other cell lines with defects in the DDR, such as BRCA1, failed to promote cisplatin resistance suggesting that CHD4 depletion mediates resistance specifically in BRCA2 mutant cells (Figures 3-13, 3-14, 3-15). Consistent with CHD4 modulating therapeutic response in a BRCA2 mutant background, we identified that derived cisplatin resistant BRCA2 mutant clones have low CHD4 protein expression (Figure 3-22). In addition, reduced CHD4 mRNA levels in BRCA2 mutant ovarian tumors correlates with poor patient response and overall survival (Figure 3-27). Interestingly, CHD4 depletion does not appear to mediate genetic reversion of *BRCA2* or restoration of RAD51 based HR repair (Figures 3-7, 3-8). CHD4 depletion in BRCA2 mutant cells likely occurs by a novel mechanism that is currently still under investigation and will be discussed in depth in the final conclusions and discussion chapter IV.

Given the roles of CHD4 and other NuRD complex members in the repair of DNA DSBs, it was unanticipated that CHD4 loss would rescue the cellular

sensitivity associated with defects in *BRCA2* mutant cells (Luo et al. 2000; Pegoraro et al. 2009; Smeenk et al. 2010; Sims & Wade 2011; Polo et al. 2010; Larsen et al. 2010; Urquhart et al. 2011). In our screen, however, the *CHD4* gene was the most robust candidate (Figure 3-29). CHD4 depletion led to cisplatin resistance in several BRCA2-mutant cell lines (Figures 3-3, 3-5). Notably, our screen did not identify additional NuRD complex members (Table 3-2). This result could stem from the fact that our screen was not saturating. Alternatively, CHD4 may have unique functions aside from those associated with the NuRD complex. Even though our screen did not identify other NuRD complex proteins, we independently examined two additional shRNAs targeting the DNA binding subunit of the NuRD complex, MBD2 (Lai & Wade 2011). We found that MBD2 depletion had the opposite effect of CHD4 depletion in BRCA2 mutant cells and caused reduced proliferation, reduced colony forming efficiency, and sensitivity to cisplatin (Figure 3-4). Interestingly, while CHD4 depletion had no effect on MBD2 nuclear expression, the opposite was not true. Depletion of MBD2 with either shRNA also reduced the nuclear expression of CHD4, yet as mentioned MBD2 depletion did not mediate resistance to cisplatin in this system (Figure 3-4). This finding suggests that aside from CHD4, the integrity of the remaining NuRD complex members must still be intact in order for CHD4 depletion to mediate cisplatin resistance.

In *C.elegans* and mammalian cells that are BRCA2 proficient, CHD4 loss

reduces proliferation, increases basal levels of γ H2A.X, and increases cellular sensitivity to agents that induce DNA DSBs (Smeenk et al. 2010; Larsen et al. 2010; Polo et al. 2010). These outcomes could stem from the fact that CHD4 depletion disrupts the G1 to S cell cycle transition and causes defects in the DNA damage induced G2/M checkpoint (Smeenk et al. 2010; Polo et al. 2010). To directly address the consequences of CHD4 loss relative to BRCA2, we examined CHD4 depletion in BRCA2 proficient and BRCA2 mutant cells. Upon CHD4 depletion in BRCA2 proficient cells, we similarly observed an increase in basal levels of γ H2A.X, defects in proliferation, and sensitivity to agents that induce DNA damage (Figures 3-9, 3-10, 3-11, 3-12). This not only corroborates previously published findings, but also functionally validates that my shRNAs targeting CHD4 produce similar outcomes in the DDR in a BRCA2 proficient background.

In contrast, the phenotypes generated by CHD4 depletion were ameliorated in BRCA2 mutant cells that retain a truncated form of BRCA2. In BRCA2 mutant cells, CHD4 depletion did not affect colony-forming efficiency or induce γ H2A.X (Figures 3-11, 3-12). Moreover, CHD4 depletion in BRCA2 mutant cells enhanced resistance to not only cisplatin, but also the PARP inhibitor Olaparib, and the DNA DSB mimetic Zeocin (Figures 3-3, 3-10). One possible explanation for this phenomenon is that wild-type BRCA2 may normally function to activate the DDR upon loss of CHD4. If BRCA2 indeed functions

downstream of CHD4, this could explain why CHD4 depletion does not activate the DDR in the context of BRCA2 mutations. Given that truncated forms of BRCA2 are expressed in PEO1 and FA-D1 cells, this would also suggest that activation of the DDR upon CHD4 loss could be mediated through the far C-terminal end of BRCA2.

However, the idea that BRCA2 depletion could rescue problems associated with CHD4 depletion was not supported by our subsequent experiments. In particular, depletion of BRCA2 in concert with CHD4 depletion failed to alleviate the negative consequences of CHD4 depletion in C4-2 and U2OS cells and instead caused toxicity (Figures 3-17, 3-18). We also further explored if the truncated BRCA2 species present in PEO1 cells was necessary for general viability or for the cisplatin resistance phenotype generated by CHD4 depletion. Surprisingly, in untreated PEO1 cells, depletion of the truncated BRCA2 species induced a signaling response scored by induction of γ H2A.X (Figure 3-19). This indicates that the truncated species contains some residual function that allows cells to cope with endogenous naturally occurring forms of genotoxic stress. However, the increase in γ H2A.X in BRCA2 depleted PEO1 cells did not correspond with an increase in sensitivity to cisplatin when compared to non-silencing control cells (Figure 3-19). This could be due to the function of an alternative repair pathway that could possibly compensate for loss of the truncated species in response to agents that directly induce DNA damage.

Interestingly, co-depletion of truncated BRCA2 and CHD4 in PEO1 cells partially negated the cisplatin resistance phenotype generated by CHD4 depletion alone (Figure 3-19). This indicates that the mechanism of cisplatin resistance conferred by CHD4 depletion is at least partially dependent on the truncated BRCA2 species, which may gain function upon CHD4 depletion.

Given that the cisplatin resistance mediated by CHD4 depletion in PEO1 cells was similar to BRCA2 restored C4-2 cells we examined if CHD4 depletion promoted *BRCA2* genetic reversion to restore DNA repair by HR. Analysis of RAD51 foci formation, a traditional functional indicator of active repair by HR, in cisplatin treated cells revealed induction of RAD51 foci in BRCA2 restored C4-2 cells, but not in PEO1 cells expressing non silencing control or depleted of CHD4 with two unique hairpins (Figure 3-7). Examination of BRCA2 migration by SDS-PAGE and subsequent western blotting with an antibody raised against the BRCA2 N-terminal revealed that BRCA2 was nuclear, but still truncated in CHD4 depleted cells (Figure 3-8). The BRCA2 band in CHD4 depleted cells migrated similar to the form in PEO1 parental cells when compared to full length BRCA2 in C4-2 restored cells. Taken together, these studies suggest that CHD4 depletion in BRCA2 mutant PEO1 cells does not promote cisplatin resistance through restored BRCA2.

In BRCA2 mutant ovarian tumors, similar to the PEO1 cells used in our

study, BRCA2 mutations do not typically nullify expression of BRCA2, but instead are governed by frameshifts and premature stop codons that predict truncated BRCA2 species (Table 3-1). In addition, unlike BRCA1, the promoter of BRCA2 is not thought to undergo methylation induced gene silencing (Collins et al. 1997; Matros et al. 2005). Using mRNA derived from BRCA2 mutant tumors and information regarding patient response to therapy in the clinic we found that elevated CHD4 mRNA levels significantly correlated with improved patient response to therapy. We also found that elevated CHD4 mRNA levels correlated with improved overall survival in patients (Figure 3-27). Thus, CHD4 mRNA levels could serve as a biomarker to predict patient response to therapy specifically in BRCA2 mutant ovarian tumors.

CHD4 depletion in BRCA2 mutant PEO1 ovarian cancer cells mediated resistance to other drugs in addition to cisplatin. Our research suggests that BRCA2 mutant tumors with low CHD4 may be sensitive to other therapies. In particular, cross-examination of BRCA2 mutant cells with additional drugs identified that the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) caused toxicity selectively in BRCA2 mutant CHD4 depleted cells compared to control (Figure 3-16). Importantly, TSA treatment also caused toxicity to BRCA2 mutant derived cisplatin resistant clones that lost CHD4 expression (Figure 3-24). In the BRCA2 restored clone C4-2, CHD4 depleted cells were not selectively more sensitive to treatment with TSA as compared to control cells (Figure 3-16).

The HDAC inhibitor TSA may therefore have potential as an alternative therapy for BRCA2 mutant tumors with reduced CHD4 expression.

Interestingly, purification of the NuRD complex nearly a decade ago from several species revealed the direct association of the histone deacetylases HDAC1 and HDAC2 (Tong et al. 1998; Wade et al. 1998; Zhang et al. 1998). These subunits catalyze histone deacetylation and are thought to be associated with transcriptional repression (Tong et al. 1998; Ng & Bird 2000). However, adding to the complexity, CHD4 association with histone deacetylases and methyl transferases can also result in transcriptional activation (Shimono et al. 2003; Williams et al. 2004; Denslow & Wade 2007). Laser micro-irradiation of human U2OS cells revealed that HDAC1 is transiently recruited to DNA DSBs in a manner partially dependent on CHD4 (Polo, 2010). In this study, CHD4 depletion mediates sensitivity to the HDAC inhibitor TSA in the presence of BRCA2 mutations. We did not examine if CHD4 depletion effects nuclear expression or the deacetylase activity of HDAC1 or HDAC2, but the finding that TSA treatment is toxic to CHD4 depleted cells suggests that these HDAC dependent processes may still be functional and aid in the survival of BRCA2 mutant cells upon loss of CHD4.

In vivo studies in a BRCA2 mutant mouse model of ovarian cancer would be best suited to determine if TSA has therapeutic potential in tumors with

reduced CHD4 expression. To date, a BRCA1 mutant mouse model of ovarian cancer exists that is also driven by loss of the p53 tumor suppressor (Xing & Orsulic 2006). However, our study demonstrates that CHD4 depletion mediated toxicity in BRCA1 mutant cells and CHD4 expression levels had no correlative effect on progression free survival in BRCA1 mutant tumors (Figures 3-13, 3-14, 3-26). Thus, future studies examining the therapeutic response of BRCA2 mutant tumors would greatly benefit from a mouse model that expresses mutant BRCA2 in the ovarian epithelium.

Altogether, this study provides an understanding of how *BRCA2* mutant cells can gain resistance to cisplatin independently of *BRCA2* genetic reversion and restoration of HR. Importantly, loss of CHD4 expression promotes resistance to cisplatin and other drugs and could serve as a potential biomarker to predict patient response in the clinic. Given that treatment with the HDAC inhibitor TSA causes selectively toxicity to CHD4 depleted BRCA2 mutant cells, TSA may be a potential therapeutic alternative for BRCA2 patients presenting ovarian tumors with low CHD4 expression.

Materials and Methods:

Cell Lines

Ovarian cancer cell lines PEO1, C4-4, C4-11, C4-13, and C4-2 were grown in DMEM with 10% FBS and 1% Glutmax, CAPAN1, FA-D1, XP-F, PD20, and FA-J patient cells were grown in DMEM with 15% FBS. We thank Toshi Taniguchi for PEO1 series cells and Orlando Scharer for XP-F cells.

shRNA Screen

The human shRNAmir pGIPZ library (release 6.0; Open Biosystems) was generously obtained through the UMass Medical School shRNA library core facility. Lentiviral pools were generated and used to transduce PEO1 cells as described at an MOI of 0.2 (Gazin et al., 2007). Cells that bypassed 2 cycles of 2 uM cisplatin treatment (enough to induce ~100% lethality in shNSC pool) underwent cellular proliferation to form colonies. Colonies were pooled and expanded, and the shRNAs identified by sequence analysis as previously described (Gazin et al., 2007). For validation, individual knockdown cell lines were generated by stable transduction of 6×10^4 cells with a single shRNA.

RNA Interference

The packaging cell line 293TL was used to produce lentiviral particles containing pGIPZ or pLKO.1 vectors. Cells were transfected with 1:1:2 ug of DNA packaging versus insert using Effectene® transfection reagent (Qiagen) 48 h prior to

harvesting retroviral or lentiviral supernatants. Supernatants were filtered and added to recipient cell lines with 1 ug/ml polybrene. Cells infected with shRNA vectors were selected with either puromycin (pGIPZ, pLKO.1). For shRNA-mediated silencing the mature antisense was used for pGIPZ CHD4-1 5'-ATTCATAGGATGTCAGCAG -3', for CHD4-2 5'-TTAGTTCTGTCTTGGAGGG -3' and for pLKO.1 CHD4-1 5'-GCTGCTGACATCCTATGAATT-3', for CHD4-2 5'-GCTGACACAGTTATTATCTAT-3'.

Immunofluorescence

Cells were fixed for 10 min with 3% paraformaldehyde/2% sucrose in phosphate buffered saline (PBS) and permeabilized for 5 min with 0.5% Triton X-100. Coverslips were rinsed 3x in 1xPBS prior to each step. For cocktails of primary and secondary antibody staining, cells were incubated for 40 min. each in a humid chamber, face down on a 100uL meniscus of Abs diluted in 3%BSA in PBS. Primary Abs used were anti-RAD51 (1:500 X), anti-gamma H2AX (1:500 Y). Secondary Abs used were Rhodamine Red-X conjugated AffiniPure Goat anti-rabbit IgG and fluorescein (FITC)-conjugated AffiniPure Goat anti-mouse IgG (Jackson Immuno-research Laboratories Inc). Coverslips were mounted on slides using Vectashield® mounting media with DAPI (Vecta laboratories, Inc) and analyzed on a fluorescence microscope (Leica DM 5500B) with a Qimaging Retiga 2000R Fast 1394 camera.

Western Blot

Cells were harvested and lysed in 150 mM NETN lysis buffer (20mM Tris (pH 8.0), 150 mM NaCl, 1mM EDTA, 0.5% NP-40, 1mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail) for 30 minutes on ice. Cell extracts were clarified by centrifugation at 14,000 rpm, protein was quantified by Bradford assay, and lysates were boiled in SDS loading buffer. Nuclear extracts were prepared with a Nuclear Extraction Kit (ThermoFisher) and Proteins were separated by SDS-PAGE on 4-12% bis Tris or 3-8% Tris Acetate gels (Novex, Life Technologies) and electrotransferred onto nitrocellulose membranes. For cellular fractionation used for immuno-precipitation, cells were pelleted and the cytoplasmic fraction was removed with incubation for 5 minutes in CSK buffer. The remaining pellet was washed 2x in CSK buffer and then re-solubilized in 150 nM NETN and sonicated with 3- 2 second pulses. The lysate was collected as the nuclear fraction after centrifugation and was pre-cleared for 1 hr at 4 degrees Celsius with protein A beads prior to Bradford analysis, overnight immune-precipitation, and SDS-PAGE. Membranes were blocked in 5% milk diluted in PBS. Antibodies used for Western blot analysis included anti-Bactin (1:5000 Sigma), anti-MCM7 (1:1000 Abcam), anti-CHD4 (1:1000 Abcam), anti-MBD2 (1:500 Santa Cruz), anti-BRCA2 N-terminal ab (1:1000 Abcam), anti-Rad51 3C10 (1:500 Millipore), anti-Rad51 H-92 (1:1000 Santa Cruz), anti-phosphS138 H2A.X (1:500 Bethyl), and anti-Cleaved Caspase-3 Asp175 (1:500 Cell Signaling). Membranes were washed and incubated with horseradish

peroxidase-linked secondary antibodies (Amersham 1:5000), and detected by chemiluminescence (Amersham).

FACS Analysis

Cells were cultured to ~ 60% confluency were left untreated or treated with 0.5 uM Cisplatin before collecting and washing with 1x PBS and fixing with 90% methanol. Cells were labeled with propidium iodide prior to analysis on a FACS Caliber flow cytometer (Becton-Dickinson) performed at the University of Massachusetts Medical School flow cytometry core facility using Cellquest software.

DNA constructs

GFP-CHD4 was expressed in PEO1, C4-2, and C4-4 cells as described in (Polo, 2010) and was a generous gift from Stephen Jackson. Cells were harvested 48 hours after transient transfection for analysis of GFP expression by western blotting.

Statistics

All quantitative data were collected from experiments performed in triplicate, and expressed as mean \pm s.d. Differences between experimental groups were calculated using a two-tailed, unpaired student t-test using Excel Microsoft or

Graphpad. Where shown, asterisks denote significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$.

CHAPTER IV:

Final Summary and Discussion

In my series of investigations dissecting the roles of tumor suppressor proteins in the DDR I have revealed (I) a novel function for the DNA helicase FANCD1 in protecting the genome against UV irradiation and (II) a role for the chromatin remodeling factor CHD4 in modulating the cellular response to DNA damage in BRCA2 mutant cells. Though these two separate investigations were not directly linked, they provide a better understanding of how tumor suppressor proteins function in the DDR.

In my first investigation I identified that both NER and MMR proteins support the localization of FANCD1 at sites of UV-induced lesions to ensure a checkpoint response during S phase of the cell cycle (Figures 2-2-2-5, 2-7-2-10, 2-15-2-19). The MMR proteins initially recruit FANCD1 to DNA lesions and further accumulation requires dual incision by the NER endonucleases XPF and XPG (Figures 2-2-2-5, 2-7-2-10). Consistent with FANCD1 and MMR functioning in a common pathway, I found that FANCD1 or MMR deficiency alone or in combination generated similar defects in the checkpoint response and in the elimination of UV induced DNA lesions (Figure 2-20). Interestingly, FANCD1 depletion did not result in UV-induced toxicity. However, my analysis revealed an important role for FANCD1 in suppressing UV-induced mutations, especially C>T

point mutations, the most prevalent type of mutation found in melanoma (Figure 2-26, Table 2-1) (Drobetsky et al. 1987; Berger et al. 2010; Hodis et al. 2012).

By searching sequenced melanoma genome databases such as cBioPortal and the Catalogue of Somatic Mutations in Cancer (CoSMiC) I discovered mutations in several proteins investigated in my functional assays, namely FANCI, MSH2, MSH6, MLH1, and PMS2 (Table 2-2) (Cerami et al. 2012; Gao et al. 2013; Forbes et al. 2011). Importantly, while about 5.7 % of the melanomas carried heterozygous FANCI mutations, these tumors did not co-segregate with the MMR associated mutations (Cerami et al. 2012; Gao et al. 2013). Given that my co-depletion studies of FANCI and MSH2 suggested these factors function in a common pathway in response to UV irradiation, the finding that MMR and FANCI associated mutations do not co-segregate supports this notion and suggests that “one-hit” could be sufficient to inactivate this pathway.

The majority of FANCI melanoma associated mutations target important domains including the helicase domain, the Fe-S domain, and helicase boxes III, IV, and V (Figure 2-29). Importantly, some of the mutations have been detected previously in other types of cancer. The FANCI^{P47} amino acid change was also targeted in hereditary breast cancer and was demonstrated to be ATPase and helicase inactive in *in vitro* assays (Cantor et al. 2004). The splice variant FANCI^{R831} is the same allele found in Fanconi anemia patients and eliminates

conserved helicase boxes required for enzyme function (Cantor et al. 2004; Cantor & Guillemette 2011; Gupta et al. 2005).

Although I did not examine the FANCD1 melanoma associated mutations in functional assays, since the majority of these mutations targeted the ATPase/helicase domain I ectopically expressed the catalytically inactive FANCD1^{K52R} mutant in U2OS cells. Overexpression of the FANCD1^{K52R} mutant determined that loss of FANCD1 ATPase/helicase/translocase activity disrupts the clearance of UV induced lesions in a dominant negative manner. Interestingly, FANCD1^{K52R} expression had no significant effect on RPA phosphorylation at the time point examined (Figure 2-30). Therefore, while expression of the enzyme inactive mutant dominantly disrupted lesion repair, it appears that loss of FANCD1 expression is required to disrupt checkpoint activation in response to UV irradiation.

Future studies that express FANCD1 melanoma associated mutants in non-transformed melanocytes would be informative to determine if these FANCD1 species truly destabilize the genome to support melanoma transformation in the face of UV irradiation. CRISPR/Cas-mediated genome editing could be used to express FANCD1 melanoma associated mutations and examine transformation potential in melanocytes (Cong et al. 2013). It would also be interesting to examine FANCD1 melanoma associated mutations and the latency of melanocyte

transformation in the context of oncogenic BRAF or RAS mutations, given the large number of melanomas harboring mutations in these genes (Brose et al. 2002; Davies et al. 2002; Omholt et al. 2003; Pollock et al. 2003; Wan et al. 2004). Paradoxically, BRAF^{V600E} mutations are also present in benign melanocytic nevi and have been shown to induce cellular senescence (Pollock et al. 2003). This brings attention to the fact that additional genetic alterations must be required in order to promote cellular transformation. A loss of function screen performed to identify factors that could escape BRAF^{V600E} induced senescence did not identify FANCI, but the screen was not saturating (Wajapeyee et al. 2008). Taken together, although my findings support the notion that FANCI could be a suppressor of cancers induced by UV irradiation, the function of FANCI as a tumor suppressor in melanoma still remains to be determined.

In the second part of my investigation I performed a genome wide shRNA screen to identify factors that modulate cisplatin resistance in a BRCA2 mutant background (Figure 3-2). The most robust target identified from my screen was the NuRD complex member CHD4 (Figure 3-3). Examination of CHD4 depletion in other cell lines with known defects in the DDR, such as BRCA1, did not rescue cellular sensitivity to cisplatin suggesting that rescue is specific to a BRCA2 mutant background (Figures 3-13-3-15). In addition, depletion of second unique NuRD complex member, MBD2, in BRCA2 mutant cells did not promote resistance to cisplatin (Figure 3-4). This further suggests that cisplatin resistance

in BRCA2 mutant cells is not mediated by general loss of the NuRD complex, but is specifically mediated by loss of CHD4. Importantly, in BRCA2 mutant ovarian tumors CHD4 expression could serve as a prognostic factor. Patients with high CHD4 expression have significantly improved progression free survival and improved overall survival (Figure 3-27).

As CHD4 has been shown to function in the DDR, I was intrigued to examine the loss of CHD4 in both BRCA2 proficient and BRCA2 mutant backgrounds (Smeenk et al. 2010; Larsen et al. 2010; Polo et al. 2010). *BRCA2* genetic reversion is one known mechanism that promotes cisplatin resistance in cell culture and in patients (Sakai et al. 2008; Edwards et al. 2008; Sakai et al. 2009). Using BRCA2 mutant PEO1 cells and their revertant counterpart, BRCA2 restored C4-2 cells I was able to deplete CHD4 in both backgrounds (Sakai et al. 2009). Upon examination of CHD4 depletion, I found that the negative consequences associated with proliferation and toxicity to DNA damaging agents in the BRCA2 proficient cells were ameliorated in the BRCA2 mutant cells (Figures 3-10-3-12).

These findings led me to hypothesize that BRCA2 was functioning downstream of CHD4 and activating a DDR upon CHD4 depletion. However, co-depletion of CHD4 and BRCA2 in C4-2 and U2OS cells did not alleviate the phenotypes associated with CHD4 depletion (Figures 3-17, 3-18). It is possible

that BRCA2 is not functioning downstream of CHD4 or that BRCA2 has other essential recombination-based roles required for viability that when lost promote cell death regardless of whether or not CHD4 is expressed.

I also discovered that the truncated form of BRCA2 was required for PEO1 cells to mediate cisplatin resistance upon CHD4 depletion (Figure 3-19). It is possible that the truncated form of BRCA2 may gain a new function upon CHD4 depletion. The truncated BRCA2 in PEO1 cells retains several important domains including the binding domain for the partner and localizer of BRCA2 (PALB2), four BRC RAD51 binding domains, the transcriptional activation domain, and the binding domain for the oncogene EMSY which potentially suppresses the transcriptional activation of BRCA2 (Hughes-Davies et al. 2003; Sakai et al. 2009; Jensen 2013). In response to cisplatin I confirmed that the truncated form of BRCA2 is still capable of binding RAD51 in a DNA damage inducible manner (Figure 3-29). This again suggests that truncated BRCA2 retains residual function. Experiments using an N-terminal BRCA2 antibody or an antibody raised against a tagged version of truncated BRCA2 could isolate novel species associating with truncated BRCA2 in cells isogenic for CHD4 expression. This approach could shed light on the protein interactions or potential new functions that truncated BRCA2 gains upon CHD4 depletion to mediate cisplatin resistance.

As CHD4 depleted BRCA2 mutant cells were cross-resistant to other DNA damaging agents aside from cisplatin, I further screened FDA approved drugs and discovered that these cells were selectively sensitive to the histone deacetylase inhibitor Trichostatin A (Figure 3-16). This was initially surprising given that the histone deacetylases HDAC1 and HDAC2 directly associate with the NuRD complex that also contains CHD4 (Tong et al. 1998; Wade et al. 1998; Zhang et al. 1998). Interestingly, this suggests that CHD4 depleted cells retain histone deacetylase activity despite loss of this NuRD complex member. Inhibiting histone deacetylase activity is thought to relax chromatin by allowing retention of the acetyl moieties on histones. Changes in chromatin structure upon HDAC inhibition are also likely to effect transcriptional profiles fostering re-programming of the transcriptome. Interestingly, the NuRD complex has been associated with transcriptional repression and activation (Tong et al. 1998; Ng & Bird 2000; Shimono et al. 2003; Williams et al. 2004; Denslow & Wade 2007). It is possible that CHD4 depletion could facilitate transcriptional re-programming and CHD4 depleted cells could be completely reliant on their new transcriptional program for survival. HDAC inhibition could promote toxicity in CHD4 depleted cells by perturbing their transcriptional profile. It is also possible that changes in the transcriptome of CHD4 depleted cells could be responsible for mediating the resistance to DNA damaging agents observed in the BRCA2 mutant background. To determine if the mechanism of cisplatin resistance in CHD4 depleted BRCA2 mutant cells is dependent on transcription I could transiently treat cells with

transcriptional inhibitors prior to or during the course of cisplatin treatment and analyze survival.

If CHD4 depleted cells gain resistance by a mechanism dependent on transcription, it would be useful to perform microarrays or whole transcriptome shotgun sequencing (RNA-seq) on BRCA2 mutant cells containing stable hairpins targeting a non-silencing control or CHD4. These types of experiments could yield a dozen or several hundred potential candidates that would require careful validation. As CHD4 depletion promotes cisplatin resistance in several BRCA2 mutant cell lines, RNA-seq could be performed on cells depleted of CHD4 versus non-silencing control in different BRCA2 mutant backgrounds. The resulting data sets could be cross-examined in an attempt to narrow down the list of potential candidates.

Another interesting aspect that could be explored is the transcriptional transactivation activity contained within the N-terminal of truncated BRCA2 (Fuks et al. 1998; Marmorstein et al. 1998; Nordling et al. 1998; Yoshida & Miki 2004). With a highly specific antibody, one could perform chromatin immunoprecipitation sequencing (ChIP-Seq) on BRCA2 mutant cells depleted of CHD4 versus non-silencing control. This would help determine if CHD4 depletion alters the positions of truncated BRCA2 binding in the genome. If changes occur upon CHD4 depletion, the regions bound by BRCA2 could be cross-examined with

RNA-Seq data sets generated from CHD4 “isogenic” cells mentioned above. Cross-examination of BRCA2 ChIP-Seq and RNA-seq data sets could potentially identify transcriptional changes mediated by truncated BRCA2 upon CHD4 depletion. Conversely, since BRCA2 is directly involved in DNA repair, it is likely to be ubiquitously bound to many regions of the genome. ChIP-Seq experiments performed with a BRCA2 antibody could therefore be very noisy and useless in identifying BRCA2 dependent transcriptional targets. In any case, one would first need to determine if the mechanism of cisplatin resistance in CHD4 depleted BRCA2 mutant cells is dependent on transcription before RNA-Seq or BRCA2 ChIP-Seq experiments would be warranted.

Taken together, these studies have allowed me to better appreciate the difficulties associated with isolating one gene and studying its outcome in the context of DNA repair or drug resistance. In the case of the shRNA screen I was able to identify several genes that modulate cisplatin resistance in the context of BRCA2 mutations (Figure 3-28, Table 3-2). While acquiring the list of >100 genes was exciting, it also proved to be a daunting task for validation. In these types of screens one hopes to ultimately elucidate at least some aspect of the problem they are trying to study and I feel that I have accomplished that. Whether taking a candidate approach as in my first investigation or an unbiased approach as in my second investigation, I have realized that biological processes are always extremely complex. Once I understand a specific molecular process I

am always led to question many others. What I can trust is that these curiosities will always drive research forward.

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